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Volume 123
Number 1
January 2025

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Contents

Article

- 1–12 Jennifer L. Gardner, W. Christopher Long, and Katherine M. Swiney
Reproductive potential of red king crab (*Paralithodes camtschaticus*) across warm and cold stanzas in Bristol Bay in southwestern Alaska

Note

- 13–21 Zachery Young, Sean Wilms, Walter J. Golet, and David W. Kerstetter
Postrelease survival of undersized swordfish (*Xiphias gladius*) caught on pelagic longline gear in a U.S. commercial fishery in the western North Atlantic Ocean

Articles

- 22–33 Paola G. Batta-Lona, Melissa L. Wojcicki, Max D. Zavell, Ann Bucklin, and Hannes Baumann

Using DNA metabarcoding to reveal prey diversity in diets of juvenile black sea bass (*Centropristis striata*) in Long Island Sound in the Northwest Atlantic Ocean

- 34–57 Yvonna K. Press, Richard S. McBride, Emilee K. Tholke, and Mark J. Wuenschel

A comparison of 4 histological staining methods for revealing oocyte development, atresia, and postovulatory follicles in 3 fish species

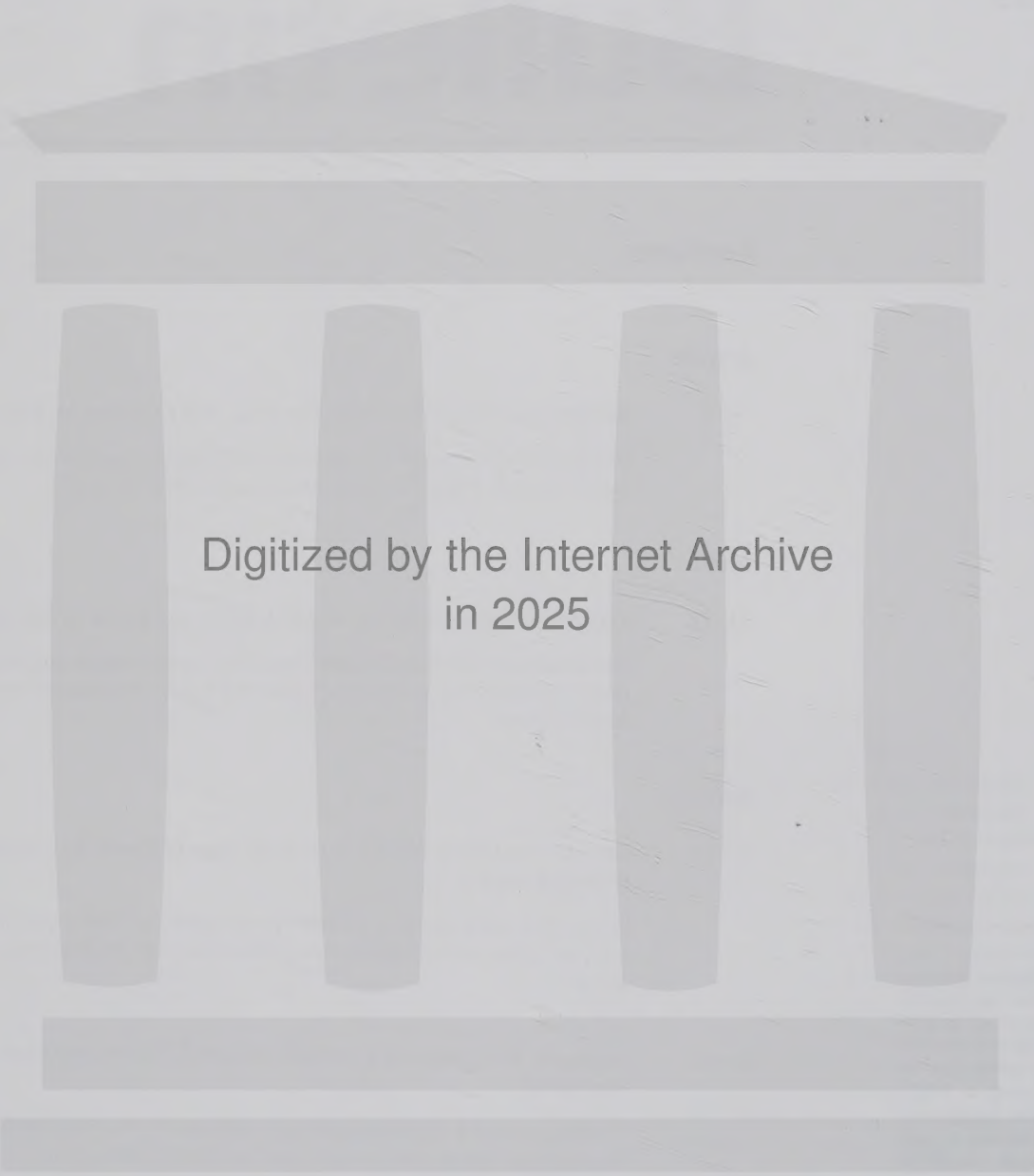
- 58–70 André L. Price, Richard S. McBride, Cara C. Schweitzer, Maurice K. Crawford, and Bradley G. Stevens

Comparing the localized feeding ecology of black sea bass (*Centropristis striata*) at natural and artificial reefs: gut content and stable isotope analyses

- 71–74 Guidelines for authors

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Abstract—The red king crab (*Paralithodes camtschaticus*) is an important commercial fishery species in Bristol Bay in southwestern Alaska. Mature male biomass is used currently in assessment of this stock as an estimate of stock reproductive potential, but assessments can be improved by directly incorporating female reproductive potential. The climate of the Bering Sea oscillates between warm and cold stanzas, which have important consequences for the ecosystem in this area. With this study, we have added to previous work quantifying the relationship between size and fecundity of females, by examining whether thermal regimes affect reproductive output. Egg clutches were collected in 8 different years spanning warm and cold stanzas, and the fecundity during each year was determined. Although we found small differences in the relationship between size and fecundity among years, the relationship did not differ between warm and cold years. Average embryo dry mass was slightly different among years but not between warm and cold years. Additionally, the relationship between residual fecundity and average egg mass was negative, indicating that some variance in fecundity is driven by trade-offs between embryo size and number. We present a size–fecundity model for Bristol Bay red king crab that could be used to estimate reproductive output from the annual survey of the population of red king crab in Bristol Bay and could be incorporated into stock assessments in the future.

Reproductive potential of red king crab (*Paralithodes camtschaticus*) across warm and cold stanzas in Bristol Bay in southwestern Alaska

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Quantifying the reproductive potential of a stock is a critical element for fisheries management. Historically one of the most valuable fisheries in the United States (Bowers¹), the fishery for red king crab (*Paralithodes camtschaticus*) in Bristol Bay, in southwestern Alaska, was closed in 2021 and 2022 because of low numbers of mature females after a massive decline. Although total female abundance is considered in the assessment and management process, this measure is coarse and fails to account for variance in egg production due to factors such as female size, age, and environmental effects on fecundity (Palof, 2023). Male reproductive potential, which is used in the stock

assessment model for Bristol Bay red king crab (Palof, 2023), refers to the number of mature males multiplied by the estimated number of females they can fertilize within a mating season. With this approach, any direct estimate of egg production is eschewed in the stock assessment for Bristol Bay red king crab (Swiney et al., 2012).

Fecundity, or embryo production, has been suggested as an improved measure of reproductive potential (Bell²; Caputi³). One of the main reasons that spawning stock biomass is used as a proxy for reproductive potential for some fisheries stocks, including for the state of Alaska's management of red king crab, is that it is easy to estimate.

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¹ Bowers, F. R., M. Schwenzfeier, S. Coleman, B. Failor-Rounds, K. Milani, K. Herring, M. Salmon, and M. Albert. 2008. Annual management report for the commercial and subsistence shellfish fisheries of the Aleutian Islands, Bering Sea and the Westward Region's shellfish observer program, 2006, 230 p. Alsk. Dep. Fish Game, Fish. Manage. Rep. 08-02. [Available from Alsk. Dep. Fish Game, P.O. Box 115526, Juneau, AK 99811-5526.]

² Bell, M. C. 2006. Review of Alaska crab overfishing definitions: 24–28 April 2006, Seattle, Washington. Report to the University of Miami Independent System for Peer Reviews, 29 p. [Available from website.]

³ Caputi, N. 2006. Alaskan crab overfishing definitions review: Seattle, Washington, 24–28 April 2006, 17 p. Report by the University of Miami Center for Independent Experts for the NOAA Alaska Fisheries Science Center. [Available from website.]

Clutch fullness (CF), a qualitative estimate of relative clutch size traditionally estimated for lithodid crab species (Donaldson and Byersdorfer, 2005), is a poor estimator of relative fecundity (Swiney et al., 2010). To incorporate female reproductive potential into the management process, it is essential to quantify the functional relationships affecting embryo production and any underlying variability in fecundity (Swiney et al., 2012) due to female size, reproductive status, interannual variability, or environmental variability.

In Bristol Bay, male red king crab mate with soft-shelled females over a 5-month period that varies interannually but usually occurs from the end of January through June (Otto, 1990). Females must mate each year to produce an egg clutch as they cannot store sperm (Powell and Nickerson, 1965). Females molt and, within a span from hours to a few days, extrude eggs, which males fertilize externally; these embryos are brooded for 10–12 months prior to hatching (Powell and Nickerson, 1965; Stevens and Swiney, 2007). Fecundity of red king crab increases with female size (Otto et al., 1990), although there is evidence of declining fecundity as females approach senescence (Swiney et al., 2012). Fecundity is also lower in primiparous females, those brooding their first clutch of embryos, than in multiparous females, those brooding their second or later clutches (Swiney and Long, 2015). Although interannual variability in fecundity is low (Swiney et al., 2012), the potential effects of thermal variability remain unknown. Several aspects of the reproductive biology of red king crab, including the timing of molting and mating, duration of embryonic development, and ultimately hatch timing, depend on presiding water temperatures (Shirley et al., 1990; Stevens et al., 2008). In years with colder than average bottom water temperatures, embryonic development is prolonged and hatching and mating occur at later dates for multiparous females (Stevens et al., 2008; Chilton et al., 2010; Foy and Armistead, 2013).

In the Bering Sea, climatic oscillations occur at a decadal scale. Since the early 2000s, the eastern Bering Sea (EBS) has undergone multiyear stanzas characterized by either warm bottom water temperatures associated with low winter ice coverage (warm years or stanzas) or cold bottom temperatures with widespread winter ice coverage (cold years or stanzas) (Stabeno et al., 2012a, 2018). Mechanistically, the strength and position of the Aleutian low pressure system, tied to the Pacific Decadal Oscillation, influences winds and surface heat fluxes in the Bering Sea, in turn, affecting the formation, advection, and persistence of sea ice across the continental shelf of the Bering Sea (Stabeno et al., 2001) and the formation and extent of the cold pool (bottom water temperature $<2^{\circ}\text{C}$). These oscillating climate stanzas affect the ecosystem, altering primary production and food supply to both pelagic and benthic communities, causing spatial shifts in stock distribution, and changing growth and molt timing in commercial fish and crab species (Orensanz et al., 2004; Mueter and Litow, 2008; Stabeno et al., 2012b; Copeman et al., 2021). Although the bottom water temperatures in Bristol Bay during warm years are well within the

thermal tolerance limits of female red king crab and their embryos (Nakanishi, 1987; Shirley et al., 1990), it is conceivable that maternal reproductive investment could differ between these thermal stanzas because of either direct effects of temperature on crab energetics or indirect effects of food availability.

Previous studies of interannual variability in fecundity for female red king crab occurred solely in cold years in the EBS (Swiney et al., 2012; Swiney and Long, 2015). In our study, we compared previously published data on fecundity of Bristol Bay red king crab from cold years (2007–2012) with newly collected data from a set of warm years (2014–2018) (Stabeno et al., 2012a; Stabeno and Bell, 2019). This work allowed us to assess whether large-scale thermal stanzas lead to differences in reproductive investment by female red king crab in Bristol Bay, in terms of changes either to the relationship between size and fecundity or to embryo size, and allowed us to examine if trade-offs between embryo size and fecundity have differed among warm and cold years. In addition, we examined if and to what degree CF can be used to predict relative fecundity. The results of this study provide improved inputs needed to develop a more accurate model of stock reproductive potential based on female fecundity for Bristol Bay red king crab.

Materials and methods

For the purposes of this study, we classified years prior to 2014 as cold years and the years 2014–2018 as warm years on the basis of the extent of the cold pool during the bottom-trawl survey conducted in the EBS by the National Marine Fisheries Service (NMFS) (Fig. 1) and the mean bottom temperature experienced by ovigerous red king crab in Bristol Bay (weighted mean temperature) and recorded during the summer NMFS EBS bottom-trawl survey. Bristol Bay is defined in our study as king crab Registration Area T, which is the area south of $58^{\circ}39'\text{N}$, north of $58^{\circ}36'\text{N}$, and east of 168°W established for vessel registration and fishing regulation (Registration . . . 2024). This area comprises 54,536 square nautical miles (nmi^2) and, for the NMFS EBS bottom-trawl survey, is separated into 136 stations, with each station having an area of approximately 400 nmi^2 (Zacher et al., 2023). In cold years, the cold pool extended into Bristol Bay, and the weighted mean temperature was below 4°C (Table 1). Annual weighted mean bottom temperatures experienced by female red king crab in Bristol Bay were calculated by weighting station bottom temperatures by the mature female catch per unit of effort at that station and averaging this value across stations with a positive catch of red king crab (Table 1).

Egg clutches of ovigerous red king crab were collected from Bristol Bay (Zacher et al., 2023) during the NMFS EBS bottom-trawl surveys conducted in summer (Lang et al., 2019). Collection methods in 2014, 2016, and 2018 mirrored those used in 2007–2010 and in 2012 and described in Swiney et al. (2012). In summary, ovigerous

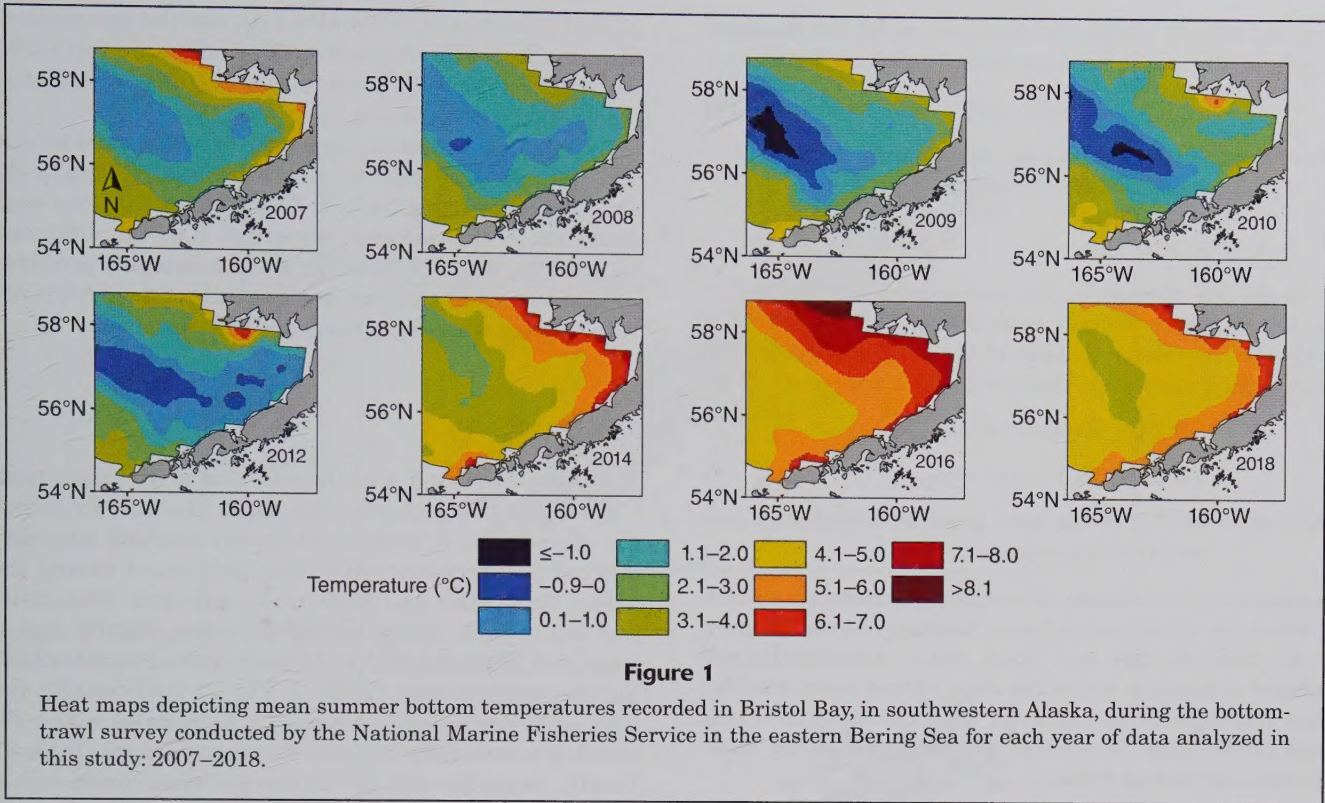


Table 1

Number of female red king crab (*Paralithodes camtschaticus*) sampled from Bristol Bay, in southwestern Alaska, during bottom-trawl surveys from 2007 through 2018 and mean bottom water temperatures at survey stations weighted by female abundance. Females were sampled to generate length-based fecundity estimates.

Year	No. of samples	Mean temperature (°C)
2007	135	3.04
2008	147	3.26
2009	133	2.67
2010	137	3.24
2012	145	2.80
2014	151	4.62
2016	146	5.33
2018	126	4.56

females were haphazardly sampled from the bottom-trawl survey until 20 clutches of recently extruded embryos (i.e., no eyes were visible on the eggs) per 10-mm size bin, with carapace lengths (CLs) between 70 and 140 mm, were collected. Twenty clutches were also collected from females with a CL greater than 150 mm. Samples were frozen at sea. For each female, CL and CF were recorded. Clutch fullness was assessed and visually approximated on a 6-point scale: 6 for a 100% full clutch,

5 for a clutch 75% full, 4 for a clutch 50% full, 3 for a clutch 25% full, 2 for a clutch within the range from a trace of eggs to a clutch 12.5% full, and 1 for a mature crab with no eggs. Although egg volume is a continuous parameter, at-sea survey work necessitates a quick grading process for all ovigerous females encountered, and CF is converted to a categorical variable through visual approximation by the sampler. Mature crab with no eggs were differentiated from immature females on the basis of their ventral anatomy (i.e., the size of their abdominal flap) per Donaldson and Byersdorfer (2005). In immature females, the abdominal flap is small and the coxae of the walking legs are exposed, making immature females easy to distinguish from barren mature crab, whose abdominal flap entirely covers the first coxa. A total of 1118 female red king crab were sampled across 7 years and ranged in CL from 73 to 167 mm.

We estimated fecundity in the laboratory by using modified protocols from Otto et al. (1990). We gently stripped embryos off the pleopods, counted 2 random samples of 250 embryos, and dried these and the remaining embryos at 60°C until a constant mass was attained. Fecundity was calculated by dividing the total embryo dry mass by the average of the 2 individual estimates of embryo dry mass from the subsamples taken to determine total embryo count.

We estimated parameters for a series of models from the fecundity data by using maximum likelihood estimation and assuming a normal distribution of errors in the statistical program R, vers. 4.2.1 (R Core Team, 2022). Normality was checked by examining a histogram of residuals

from the best-fit model. For the base model, we assumed that fecundity would increase linearly with crab size:

$$F = mCL + b, \quad (1)$$

where F = fecundity in number of embryos;
 CL = carapace length in millimeters;
 m = the slope; and
 b = the intercept.

To model the change in the size–fecundity relationship due to senescence (Swiney et al., 2012), we incorporated a split-point regression in some of the models in which the slope of the relationship changes above a critical size:

$$\text{If } CL < Sp, \text{ then } m = m_1, \text{ or} \quad (2)$$

$$\text{If } CL \geq Sp, \text{ then } m = m_2, \quad (3)$$

where Sp = the CL at the split point in millimeters and was fit as a parameter.

To examine interannual differences, we included models in which the slope was a linear function of year (Swiney et al., 2012; Swiney and Long, 2015). Additionally, we included a model in which the slope in cold years was the same (2007–2012) and differed from the slope in warm years (2014–2018). To account for differences in CF, models were included as follows:

$$F = (mCL + b)(1 - D_{CF}), \quad (4)$$

where D_{CF} = a linear function of CF and represents the percent decrease in fecundity compared to that of a full (CF=6) clutch (i.e., $D=0$ for a CF of 6).

Because of the low sample size for females with CF of 1–3 (sample size $[n]=10$), we combined data for females with CF of 2–4 for analyses.

Post hoc, we observed that slopes were similar among multiple years (2007, 2009, 2014, 2016, and 2018), as well as between 2010 and 2012. Therefore, we constructed an additional model in which those groups of years had identical slopes. Also, we observed that the D for a CF of 5 was indistinguishable from 0 ($D_5=0.0038$ [standard error of the mean (SE) 0.0042]); therefore, we included an additional model where D for a CF of 6 and a CF of 5 were both 0. Akaike's information criterion corrected for small sample sizes (AIC_c) was calculated for each model and used to select the best-fit model. Models with a difference between AIC_c values that was less than 2 were considered to explain the data equally well (Burnham and Anderson, 2002).

Average individual embryo dry weights were examined by using an analysis of covariance (ANCOVA) with maternal size (CL) fully crossed with year. Differences in egg mass among levels of CF were examined by using a 2-way analysis of variance (ANOVA), with year and CF as factors. All ANCOVAs and ANOVAs were done in SYSTAT⁴, vers.

13.2.01 (Grafitti LLC, Palo Alto, CA), and the assumptions of normality and homogeneity of variance were verified by using the Shapiro–Wilk test (normality) and Levene's test (homogeneity of variance).

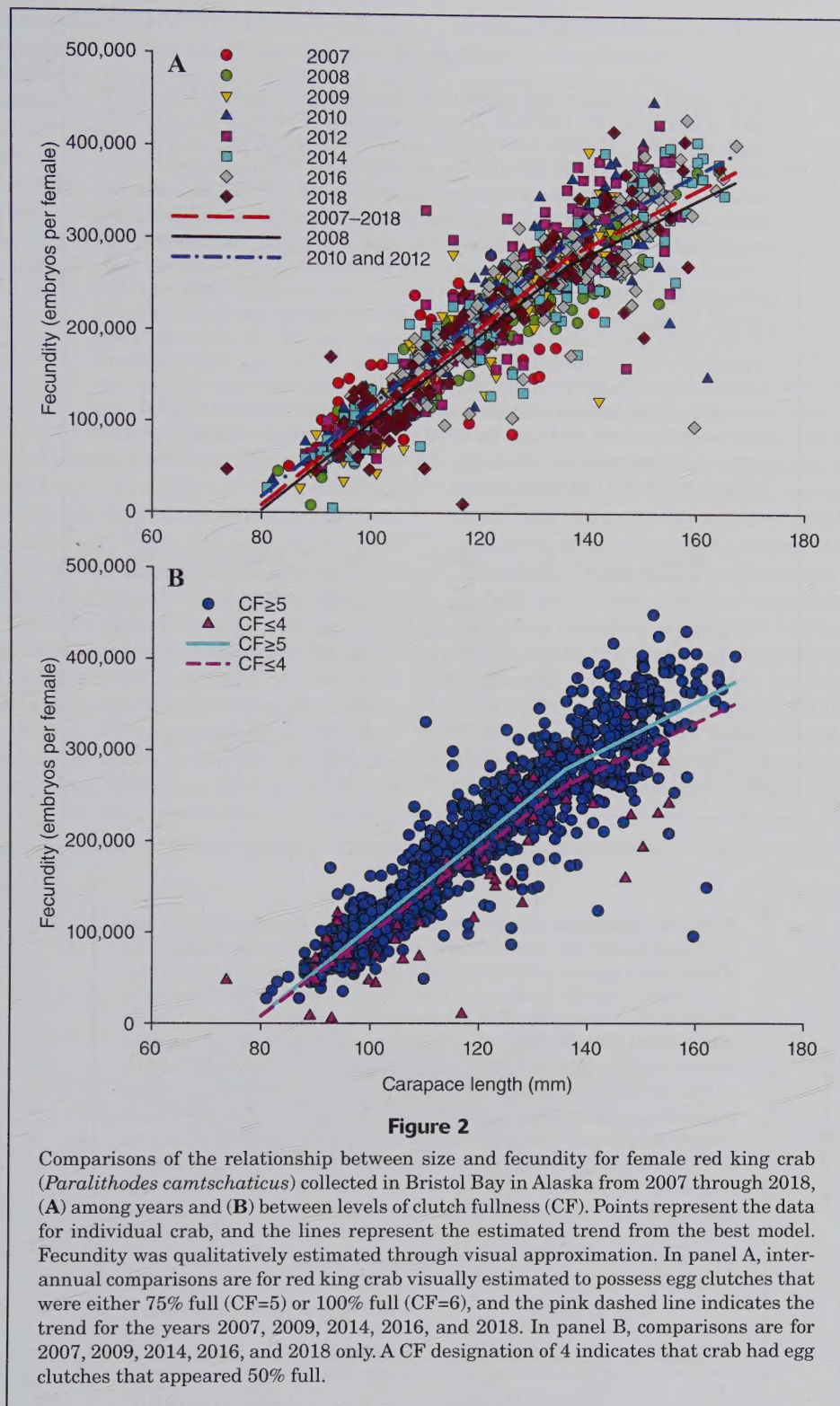
To examine potential trade-offs between embryo size and fecundity, we calculated the residual fecundity for each female by using the best-fitting model from the work described in the previous paragraph (i.e., the difference between the observed fecundity and the fecundity predicted by the model) and analyzed it with a fully-crossed ANCOVA with average embryo dry mass and year as factors.

Results

Fecundity increased with female size and ranged from 5200 to 450,000 embryos (Fig. 2). In the best-fit model, the slope of the relationship between size and fecundity differed among years, with 2008 (cold year) having the lowest slope, 2007 and 2009 (cold years) and 2014, 2016, and 2018 (warm years) having the same, slightly higher slope, and 2010 and 2012 (cold years) pooled together having the highest slope (Table 2, Fig. 2). Additionally, the split point was allowed to vary, and for females with a CL above approximately 136 mm, the slope dropped (Table 3). Finally, fecundity was 6.5% lower for individuals with a CF of less than 5 compared to that for those with a CF of 5 or above, but there was no significant difference in fecundity between individuals with CFs of 5 and 6. There was no support for the model in which slopes differed between warm and cold years (Table 2). Differences in the size–fecundity relationship among years were small (Fig. 2, Table 3). The best model proved an excellent fit to the data, explaining 86% of the variance (Table 3).

To explicitly analyze the effect of temperature on fecundity, we regressed the slope estimates for each year (from the model in which the parameter slope is allowed to vary above a split point and among years and the other parameters are intercept and standard deviation; Table 2) against the weighted mean bottom temperature. The weighted mean temperature was derived by using data from the stations of the NMFS EBS bottom-trawl survey in Bristol Bay. Temperature had no effect on the slope of the size–fecundity relationship (linear regression: $P=0.55$; Suppl. Figure [online only]). Although results from the best-fit model indicate clear differences in fecundity among the years for which data were examined, those differences were not caused by temperature and cannot be predicted from survey data analyzed in this study. To create a model that can be used to calculate estimated egg production from survey data, we fit a model that contained all the parameters in the best-fit model except those specific to year. Parameters for this model are provided in Table 3. Although the difference in AIC_c between this model and the best-fit model was 40.9 (indicating strong statistical support for differences among years), the difference in the coefficient of multiple determination (R^2) between models was only 0.006, indicating that differences among years account only for 0.6% of the variance in the data.

⁴ Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.



Mean embryo dry mass was 201 μg (SE 15) across all years. Although there was no pattern in variation of embryo dry mass with temperature stanzas, average embryo mass differed among years (ANCOVA: $F=2269$, $P=0.027$) with a 10% maximum difference (Fig. 3, Suppl. Table [online only]). Year and female size had an interactive

effect (ANCOVA: $F_{7,1104}=2.32$, $P=0.024$), indicating a violation of the assumption of homogeneity of slopes. Therefore, a linear regression between female CL and embryo mass was performed for each year separately. Embryo weights decreased with increasing maternal size for 3 of 8 years (2009, 2012, and 2016: $P<0.03$; Fig. 3) but not for the other

Table 2

Models used to examine the relationship between size and fecundity for the stock of red king crab (*Paralithodes camtschaticus*) in Bristol Bay in southwestern Alaska. Akaike's information criterion corrected for small sample sizes (AIC_c) was used to identify the best-fit model. The difference in AIC_c between each model and the best model (ΔAIC_c) and AIC_c weights for each model are also presented. K indicates the number of parameters in each model. The model parameters include the slope (m), intercept (b), decrease in fecundity as a linear function of egg clutch fullness (D_{CF}), and standard deviation (σ). Models were fit to data from bottom-trawl surveys conducted in the eastern Bering Sea during 2007–2018. In several models, the slope is allowed to vary above a split point (Sp) or to vary among years (Y). In one model, slope is allowed to vary with the thermal regime (Y_T ; i.e., the slope differs between warm and cold years). Y^* indicates post-hoc models in which the slope parameter did not differ among groups of years (data for 2007, 2009, 2014, 2016, and 2018 were the same, and data for 2010 and 2012 were the same). D_{CF}^* indicates a post-hoc model in which D_{CF} did not differ between crab with clutch fullness scores of 6 (100% full clutch) and 5 (75% full clutch).

Model	K	AIC_c	ΔAIC_c	Likelihood	AIC_c weight
m, b, σ	3	26,566.33	122.73	0.00	0.00
$m(Sp), b, \sigma$	5	26,529.35	85.75	0.00	0.00
$m(Y), b, \sigma$	10	26,536.10	92.50	0.00	0.00
$m(Y, Sp), b, \sigma$	12	26,498.65	55.05	0.00	0.00
$m(Y_T, Sp), b, \sigma$	6	26,528.45	84.85	0.00	0.00
$m(Y^*, Sp), b, \sigma$	7	26,488.38	44.78	0.00	0.00
$m(Y^*, Sp), b, D_{CF}, \sigma$	9	26,444.89	1.29	0.52	0.34
$m(Y^*, Sp), b, D_{CF}^*, \sigma$	8	26,443.60	0.00	1.00	0.66

Table 3

Parameter estimates and goodness of fit for the best-fit model of the relationship between size and fecundity for the stock of red king crab (*Paralithodes camtschaticus*) in Bristol Bay in southwestern Alaska and for a model in which interannual differences were excluded (this model represents the best model that can be used for predicting fecundity with survey data). Standard errors are indicated parenthetically. Parameters include intercept (b), slope (m), change in slope (Δm), carapace length at the split point in millimeters (Sp), change in slope above the split point (Δm_{Sp}), and proportional decrease in fecundity in crab with clutch fullness (CF) scores from 2 (from a trace of eggs to a 12.5% full clutch) to 4 (50% full clutch) (D_{CF2-4}). For Δm , subscripts indicate if the change in slope is for particular years (2008 or 2010 and 2012) or above the split point (Sp). Coefficients of multiple determination (R^2) were used to determine goodness of fit. Models were fit to data from bottom-trawl surveys conducted in the eastern Bering Sea during 2007–2018.

Best-fit model		Model averaged across years	
Parameter	Estimate	Parameter	Estimate
b	-380,708 (10,362)	b	-376,173 (10,316)
m	4863 (92)	m	4840 (91)
Δm_{08}	-69 (27)		
$\Delta m_{10,12}$	114 (21)		
Sp	136.0 (2.6)	Sp	137.7 (2.5)
Δm_{Sp}	-1789 (329)	Δm_{Sp}	-1790 (330)
D_{CF2-4}	-0.065 (0.009)	D_{CF2-4}	-0.066 (0.010)
R^2	0.857	R^2	0.851

5 years (linear regression: $P>0.05$). The effect of female size for the years for which CL was significant was small, with the size of the effect ranging from 5.7% to 7.5% across the range of CLs predicted (Fig. 3), and the relationship disappeared when all years were analyzed together ($P=0.105$, $R^2=0.002$) with a predicted 1.8% difference across the range of CLs. Residual fecundity decreased with average embryo mass (ANCOVA: $F_{1,1094}=64.412$, $P<0.0005$; Fig. 4) but did not differ among years ($F_{7,1094}=1.128$, $P=0.343$).

Discussion

This study included the first examination of the reproductive output of females in the stock of red king crab in Bristol Bay across both warm and cold thermal stanzas in the EBS. Although there were slight differences among years in the relationship between size and fecundity, there were no differences between warm and cold years. Likewise, the average embryo dry mass differed among years; however, thermal stanza had no effect, indicating that temperature does not influence maternal investment per offspring. Although there is interannual variability in the relationship between size and fecundity that cannot be predicted with our best model, the interannual variability over the years studied was negligible, explaining less than 1% of the variance in the data. We confirm that the size–fecundity relationship decreased as size increased,

a trend likely attributable to senescence, but we will not discuss that further because it has been explored previously (Swiney et al., 2012). Average embryo dry mass decreased with residual fecundity, indicating a trade-off between embryo quality and quantity for this species; however, this relationship did not differ among years or between thermal stanzas. Overall, these data indicate that female reproductive potential for Bristol Bay red king crab was unaffected by interannual differences in water temperatures.

For Bristol Bay red king crab, there are a few mechanisms by which temperature might affect reproductive investment. Differences in benthic–pelagic coupling between warm and cold years could alter food quality and quantity for red king crab (Ramos et al., 2003; Copeman and Parrish, 2004; Richoux et al., 2004). Benthic productivity in the Bering Sea is highly correlated with ocean temperatures. In warm years, the spring bloom occurs later, and warmer temperatures increase pelagic secondary production, decreasing export to the benthos (Walsh and McRoy, 1986; Hunt and Stabeno, 2002). In contrast, in cold years, high flux from ice-edge and pelagic phytoplankton blooms increases the food quality and quantity reaching the benthos, benthic secondary production, and ultimately food quantity and quality for benthic predators (Wassmann and Reigstad, 2011; Grebmeier, 2012; Grebmeier et al., 2015). Both the quantity and quality of diet items significantly affect growth and lipid storage in juveniles of *Chionoecetes* species (Copeman et al., 2021),

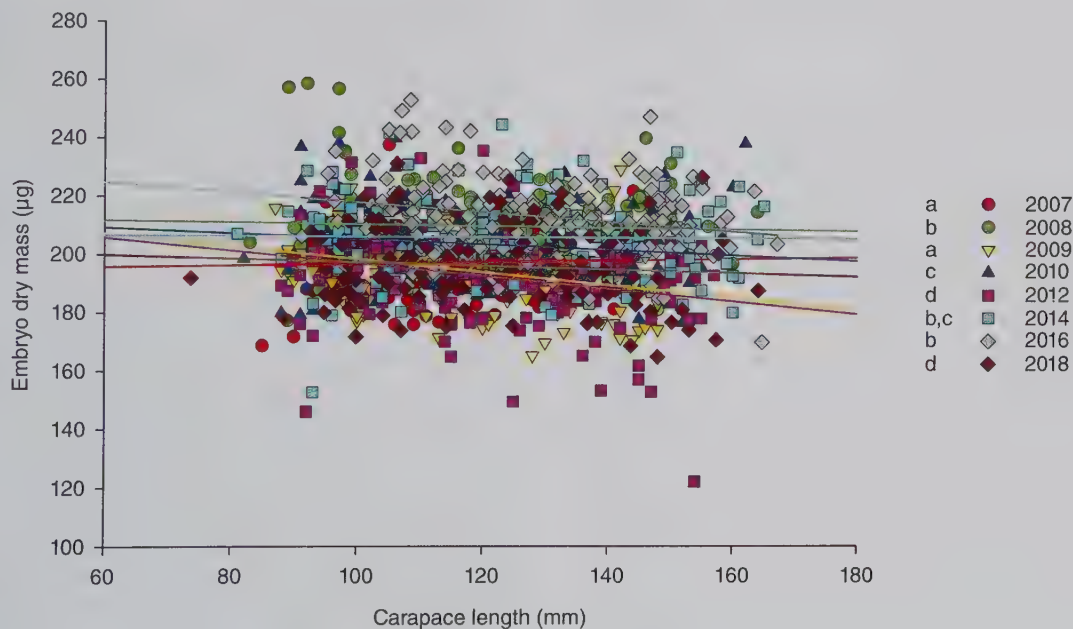


Figure 3
Interannual comparison of the relationship between maternal size and average embryo dry mass for red king crab (*Paralithodes camtschaticus*) collected in Bristol Bay in Alaska during 2007–2018. Points represent data for individual females, and the lines represent linear regressions for each year. Different letters denote significant differences in slopes.

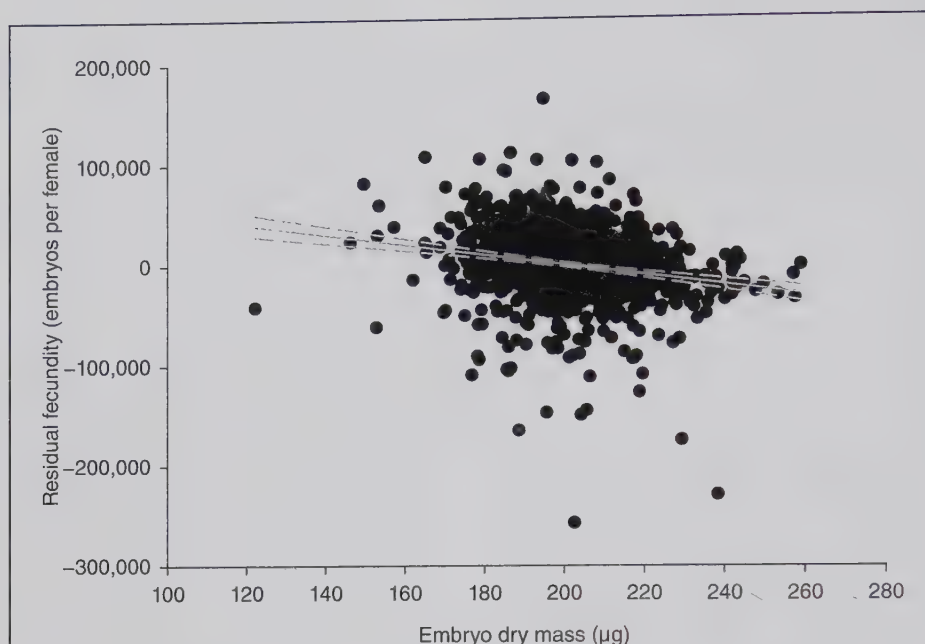


Figure 4

Plot of residual fecundity versus embryo dry mass for red king crab (*Paralithodes camtschaticus*) collected in Bristol Bay in Alaska during 2007–2018. Points represent data from individual females. The solid line represents the best-fit linear regression (coefficient of multiple determination=0.05, $P<0.0005$), and the dashed lines represent the 95% confidence bands. Residual fecundity values were calculated from the best-fit model of the relationship between size and fecundity.

and a similar response may be expected for ovigerous females of red king crab, resulting in reduced energy availability for reproduction.

A second mechanism that could cause differences in reproductive investment between warm and cold years is the direct effect of temperature on basal metabolic rates (Mauro and Mangum, 1982; Brill et al., 2015; Piatt et al., 2020). Reproductive processes, such as gonadal growth, gamete maturation, vitellus production, and aerating and cleaning the clutch, are all energetically demanding (Baeza and Fernández, 2002). Increased temperatures could raise metabolic demand and redirect energy from reproduction (Foyle et al., 1989; Pörtner et al., 2005; Copeman et al., 2021). Our data, however, offer no support for differences in reproductive investment with temperature. This result indicates that either the females were not food-limited and were able to consume more to compensate for a higher metabolic rate or, alternatively, they prioritized reproduction over other processes like somatic growth.

Future work should investigate whether there are any differences in the growth at molt or crab condition (e.g., lipid reserves) among thermal stanzas to address the second hypothesis regarding the mechanism of temperature. It is also possible that the temperatures females occupy during the NMFS EBS bottom-trawl survey are not representative of the thermal habitat occupied by ovigerous females throughout the year because of their migration

after the mating season. Current satellite tagging work on Bristol Bay red king crab combined with a new cooperative pot survey conducted in winter months in Bristol Bay will help improve understanding of the movement of females throughout the year.

Although we did not find an effect of thermal regime on the fecundity of Bristol Bay red king crab in our data, temperature plays an important role in the reproductive biology and growth of decapod crustaceans, manifesting through considerable variability in size–fecundity relationships that are specific to both species and region. The interaction of various environmental and biotic factors in different geographic areas may lead to different relationships between size and fecundity (Aiken and Waddy, 1988) or may influence interannual variation in a single area (Bagenal, 1973). The size–fecundity relationship for both American lobster (*Homarus americanus*) and European lobster (*Homarus gammarus*) varies regionally, and differences are most likely driven by temperature (Waddy and Aiken, 1991). Increases in temperature have likely caused a 23% decline in fecundity for female American lobster in southern New England due to increased egg loss (Goldstein et al., 2022).

However, a decline in fecundity is not necessarily correlated with rising temperatures. The size–fecundity relationship for the Australian spiny lobster (*Panulirus cygnus*) was the same in 2 regions with contrasting temperature extremes (Morgan, 1972). Similarly, Tully et al.

(2001) found no regional differences in the fecundity of European lobster between Ireland, the United Kingdom, and France, despite differences in temperature (they did not specifically test mean temperature as a covariate). In our study, temperatures in the warm years were still well within the thermal tolerance of red king crab and historic levels; however, if temperatures increase as predicted, it may be necessary to monitor Bristol Bay red king crab for reductions in reproductive potential. Mature red king crab from southeastern Alaska experience reduced growth at temperatures between 9°C and 12°C, and both mature females and embryos exhibit no change in mortality at temperatures up to 12°C (Shirley et al., 1990). However, thermal tolerance studies for Bristol Bay red king crab have been limited (Long and Daly, 2017). Lab-controlled studies are warranted to determine temperature thresholds for ovigerous red king crab in Bristol Bay and to determine the effects of increasing temperature on egg production and retention and on embryo development.

Embryo quality is an important component of reproduction and can directly affect offspring fitness. Allocation of energy into egg production can be both variable and adaptive, depending on the environment (Pond et al., 1996; Donelson et al., 2010; Shama et al., 2014), and embryo size is a metric of investment per offspring (Anger, 2001; Moran and McAlister, 2009). Two major resource allocations related to reproduction must be made. The first is energy allocation to reproduction (versus other metabolically demanding processes like somatic growth), and the second is energy allocation to each offspring. Two females may invest the same amount of energy into their clutches, but they will have different fecundities if their per-embryo investments differ (Giménez and Anger, 2003; Marshall and Keough, 2008).

Higher investment per embryo is often correlated with stressful environments and is likely an adaptation to increase the survival of offspring (Hartnoll et al., 2007; Jivoff et al., 2007; Firstater et al., 2012). In our study, embryo size was negatively correlated with residual fecundity for Bristol Bay red king crab (Fig. 4) but did not differ among years or thermal regimes. Although there were interannual differences in embryo weight, they did not differ among warm and cold years. For Dungeness crab (*Metacarcinus magister*), lower temperatures during embryogenesis were correlated with larger sizes at hatching (Shirley et al., 1987), highlighting the importance of examining species-specific maternal investment at various points in embryo development across time scales that capture variable thermal conditions.

Finally, this work establishes that the 6-point CF scale used on the NMFS EBS bottom-trawl survey has limited value. There was no statistical difference in fecundity at a given size between crab with 75% full clutches and crab with 100% full clutches (between crab with CFs of 5 and 6), and there was only a slight (6%) decrease in fecundity for crab with clutches that were 50% full or less (with CFs of 4 and below) in comparison to the fecundity of crab with CFs of 5 and 6, a decline that is surprisingly low given the differences in CF. Although our sample size was low ($n=8$)

for crab with CFs of 2–3, such individuals on average had 39% fewer eggs than the number of eggs predicted for a crab of the same size with a CF of 6. Two possible explanations are that individuals with a CF of 4 had smaller eggs, giving the appearance of a smaller clutch, or had a tighter packing density of their eggs. Of course, this idea would have to be substantiated through further study.

There was a significant difference in egg mass among levels of CF (2-way ANOVA: $F_{4,1098}=4.054$, $P=0.003$), with crab with CFs of 4 and 5 having smaller eggs than crab with a CF of 6. However, the effect size was small, with crab with CFs of 4 and 5 having eggs only 3.1% or 1.4% smaller than the eggs of crab with a CF of 6, respectively. These small differences are not sufficient to explain either a 25% or a 50% difference in estimated clutch volume. Mature female red king crab have a large size range, making estimation of relative versus absolute clutch size difficult and hard to standardize across multiple observers each year, and the morphology of the abdominal flap of red king crab contains no reference markers or accurate visual estimation of CF. All of these factors likely lead to substantial variation between observers in assessment of CF.

The differences in egg mass for crab with CFs of 4–6 confirm and add more data to support the idea that crab with CFs of 5 and 6 are more fecund than crab with a CF of 4 but not more than each other (Swiney et al., 2010). It is likely that the fecundity of crab with CFs of 2 and 3 is lower still (Donaldson and Byersdorfer, 2005). Crab with CFs of 2 and 3 are probably better categorized by observers than crab with higher levels of CF; however, it would take a focused study to collect sufficient crab to quantify the difference well given that only 5% of crab had a CF of 4 or below in our survey (Zacher et al., 2023).

Conclusions

The primary objective for this multiyear study of the fecundity of Bristol Bay red king crab was to develop a model of the relationship between size and fecundity that incorporates ontogenetic differences (Swiney et al., 2012; Swiney and Long, 2015) and temperature variability (work described herein) to allow estimation of total stock reproductive potential to replace the estimation of male reproductive potential that is currently used in stock assessments. We present a highly accurate model that can be used to estimate total stock egg production from data on female abundance, clutch fullness, and size frequency collected during the NMFS EBS bottom-trawl survey. If future warming results in temperatures above those observed in this study, reevaluating the effects of temperature on fecundity and maternal investment may be necessary.

In future studies, researchers should model egg production across the survey time series, identify patterns over the time series, compare egg production to past metrics for stock reproductive potential, and evaluate the implications for stock assessment. Work in the future also should use the new estimate of egg production to examine the stock–recruit relationship for red king crab to update and

perhaps improve on previous analyses (Zheng and Kruse, 2000, 2003, 2006).

Resumen

El cangrejo real rojo (*Paralithodes camtschaticus*) es una importante especie de pesca comercial en la bahía de Bristol, en el suroeste de Alaska. Actualmente, se utiliza la biomasa de machos maduros en la evaluación de esta población en la estimación de su potencial reproductivo, pero las evaluaciones pueden mejorarse incorporando directamente el potencial reproductivo de las hembras. El clima del Mar de Bering oscila entre estancias cálidas y frías, lo que tiene importantes consecuencias para el ecosistema de esta zona. Con este estudio, hemos complementado trabajos anteriores que cuantificaban la relación entre el tamaño y la fecundidad de las hembras, examinando si los regímenes térmicos afectan a la reproducción. Se colectaron lotes de desove de 8 años, abarcando temporadas cálidas y frías, y se determinó la fecundidad durante cada año. Aunque encontramos pequeñas diferencias en la relación entre tamaño y fecundidad entre años, la relación no difirió entre años cálidos y fríos. La masa seca media de los embriones varió ligeramente de un año a otro, pero no entre los años cálidos y los fríos. Además, la relación entre la fecundidad residual y la masa media de huevos fue negativa, lo que indica que parte de la varianza en la fecundidad se debe al equilibrio entre el tamaño y el número de embriones. Presentamos un modelo de tamaño-fecundidad para el cangrejo real rojo de la Bahía de Bristol que podría utilizarse para estimar la producción reproductora a partir del estudio anual su población en la Bahía de Bristol y podría incorporarse en futuras evaluaciones poblacionales.

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Abstract—Survivorship pop-up archival tags (sPATs) were attached to swordfish (*Xiphias gladius*) released from U.S. commercial pelagic longline vessels in the western North Atlantic Ocean. The purpose was to determine the postrelease survival of individuals less than the minimum retention size (119 cm in lower jaw fork length) established by the International Commission for the Conservation of Atlantic Tunas (ICCAT). Between 2021 and 2023, 24 sPATs were deployed with a programmed duration of 30 d to measure depth, temperature, and ambient light. Temperature and depth were recorded as daily minimums and maximums, and light level was recorded as a daily change between minimum and maximum values. The final 5 d of depth information were also recorded at 10-min intervals prior to release to assess fine-scale movements consistent with survival. Nineteen tags transmitted data, with 16 tags having data sufficient for analysis. Of these tags, 9 sPATs (56.2%) transmitted data consistent with movements of a surviving swordfish. This survival rate is lower than those reported for previous studies that focused on rod-and-reel and swordfish buoy gear, indicating that capture with pelagic longline gear causes swordfish greater stress than those other gear types. This result, combined with those of prior studies of at-vessel mortality rates, indicates that the current ICCAT rule regarding the minimum retention size provides limited conservation benefits to swordfish stocks.

Postrelease survival of undersized swordfish (*Xiphias gladius*) caught on pelagic longline gear in a U.S. commercial fishery in the western North Atlantic Ocean

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The swordfish (*Xiphias gladius*) is a large, highly migratory predator with a circumglobal range extending from temperate to tropical zones between 45°N and 45°S (Palko et al., 1981), although individuals have been reported as far north as 49°N in the Pacific Ocean (Haplin et al., 2018). In the western North Atlantic Ocean, adult swordfish migrate between temperate foraging grounds in summer and spawning grounds at subtropical latitudes in winter (Neilson et al., 2009; Braun et al., 2019), swimming at rates of up to 43 km/d (Canese et al., 2008). Swordfish undertake diel vertical migrations, generally staying within the mesopelagic zone during the day and moving into shallower waters to feed in the mixed layer during the night (Carey and Robison, 1981; Sedberry and Loefer, 2001; Abascal et al., 2010). These movements expose swordfish to a wide range of temperatures between 2.7°C (Braun

et al., 2019) and 31.0°C (Fenton, 2012). The movements of juvenile swordfish appear to have similar but shallower diel patterns than those of adults (Fenton, 2012; Braun et al., 2019).

U.S. fisheries that target swordfish have operated in the western North Atlantic Ocean since the 1950s, although directed harpooning by commercial fishermen can be dated to the 1800s (Ellis, 2013) and indigenous Red Paint people harvested swordfish thousands of years ago in Maine (Bourque, 2012). Responding to a substantial decline in the North Atlantic swordfish stock, the International Commission for the Conservation of Atlantic Tunas in 1990 established a minimum size for retention (125 cm in lower jaw fork length [LJFL] with a 15% allowance for incidental catches) and restricted incidental catches to no more than 10% of the entire catch (Neilson et al., 2013). Additional regulations specific to the

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U.S. fishery in the North Atlantic Ocean were later instituted, including an alternate lower retention size limit of 119 cm LJFL with a zero tolerance for retention of undersized swordfish in 1995 and a 10-year rebuilding plan involving a total allowable catch of 10,600 metric tons (t)/year (Neilson et al., 2013). Additionally, time-area closures were implemented by NOAA in 2000 to protect undersized swordfish (Neilson et al., 2013). In 2004, the use of circle hooks was made mandatory in the U.S. Atlantic pelagic longline fishery (Serafy et al., 2012). The North Atlantic swordfish stock was finally considered rebuilt in 2009 (Neilson et al., 2013).

Internationally, the fishery in the North Atlantic Ocean has had an average annual catch (landings of swordfish of a legal size combined with the number of undersized swordfish that were discarded) of around 10,400 t between 2012 and 2022 (ICCAT, 2023). The average number of undersized swordfish discarded, as a result of regulations based on a minimum size required for retention, was approximately 5844 individuals/year between 2017 and 2021 in the U.S. pelagic longline fishery alone (NMFS, 2022). Pelagic longline landings of swordfish have decreased since the 1990s because some fishermen in the U.S. waters of the Atlantic Ocean opportunistically target more valuable fish, such as tunas, or leave the fishery for economic reasons; however, swordfish are still targeted by fishermen that use buoy and rod-and-reel gears. Swordfish are also a common bycatch species in pelagic longline fisheries targeting tuna species, such as the bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*T. albacares*) (ICCAT, 2019).

Several prior studies have reported postrelease survival rates for billfishes (family Istiophoridae) released from pelagic longline gear. Specifically, the survival of blue marlin (*Makaira nigricans*) was estimated at 77.8% (Kerstetter et al., 2003), the survival of sailfin (*Istiophorus platypterus*) was estimated at 88.2% (Kerstetter and Graves, 2008), and the survival of white marlin (*Kajikia albida*) was estimated at 63.0–89.5%, depending on inclusion or exclusion of tags that did not transmit data (Kerstetter and Graves, 2006). Similar results have been reported for recreational rod-and-reel fisheries, with white marlin having 65% survival on J hooks and 100% survival on circle hooks (Horodysky and Graves, 2005). A meta-analysis across istiophorid billfishes found postrelease survival for this family to be about 86%, with most mortalities occurring from just minutes to a few days after release (Musyl et al., 2015).

Similar studies on swordfish are less common, and results have varied by gear type and size class. Tracey et al. (2023), who examined mortality of swordfish by recreational rod-and-reel gear, found that tagged adult swordfish had 86.4% survival, a rate that decreased to 44% when analysis included landed fish assessed as moribund, on the basis of a modified condition scale (Kerstetter et al., 2003) based on evaluations of overall activity, color, eyes, stomach eversion, and body musculature. However, in other studies, estimates of postrelease survival related to capture of swordfish on buoy

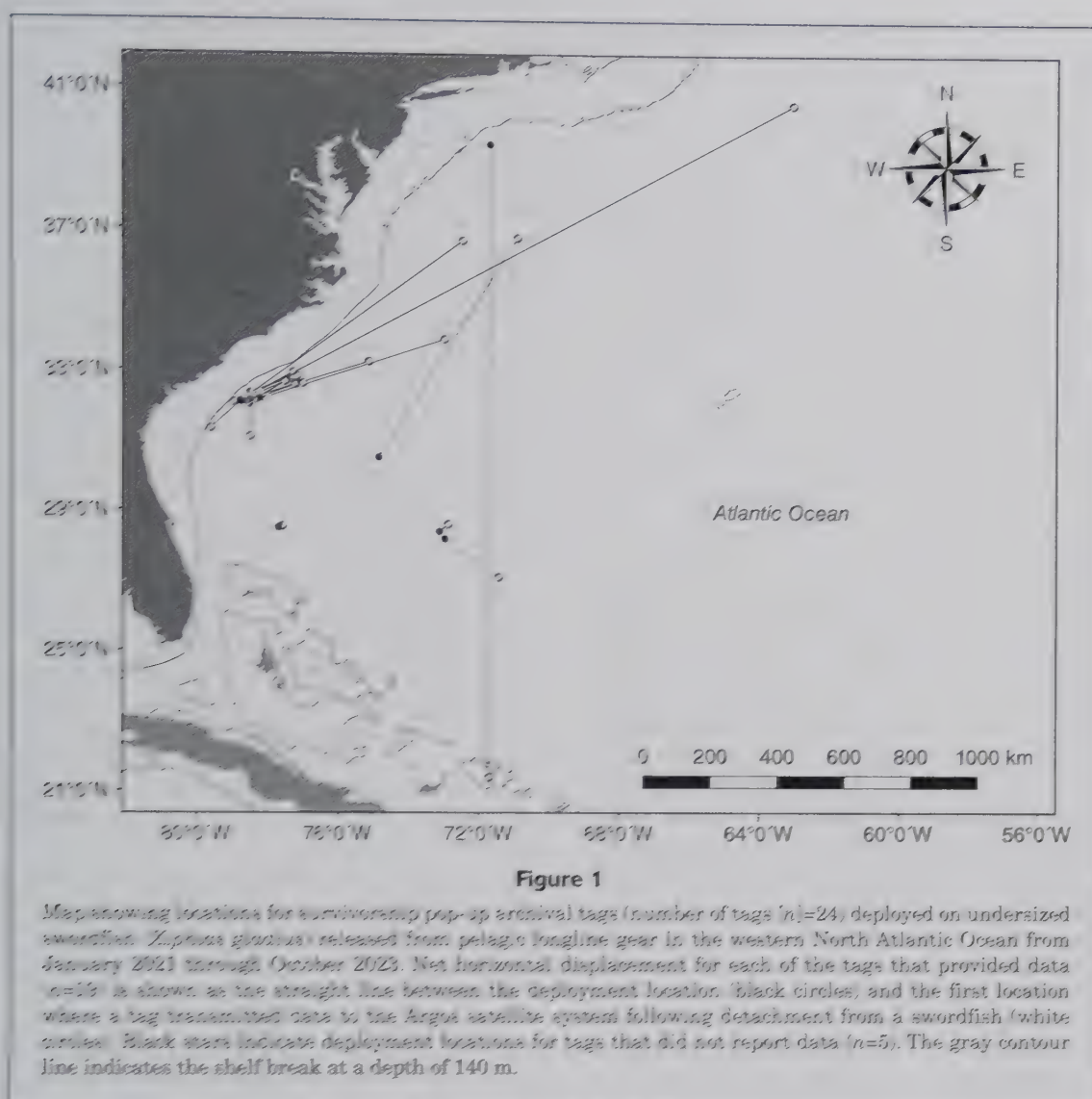
gear ranged only from 57.9% (Rewis, 2023) to 64.3% (Fenton, 2012). A high mortality of undersized swordfish on pelagic longline gear was expected considering that the at-vessel mortality (fish that are dead at gear retrieval) for undersized individuals has been estimated between 78% and 88% across the various pelagic longline fisheries in the Atlantic Ocean (ICCAT, 2019). Because no literature has been published on the postrelease survival of undersized swordfish released alive from pelagic longline gear, the primary aim of this study was to fill this knowledge gap.

Materials and methods

Survivorship pop-up archival tags (sPATs) (model 407, Wildlife Computers Inc.¹, Redmond, WA), each with a medium-sized Domeier anchor (Wildlife Computers Inc.), were used to assess postrelease survival. This electronic tag automatically activates when its conductivity sensor detects seawater and its depth sensor detects that it is submerged below a programmed depth (10 m). The sPAT weighs 61 g and measures 11.8 by 3.8 cm, minus a flexible antenna. Tags were programmed for a 30-d deployment and had predetermined early release conditions for maximum depth (1700 m), as well as for the tag remaining at a constant depth (± 2 m) or on the surface (depths of ≤ 1 m or for the wet-dry sensor indicating dry for over 50% of the time) for a period of more than 24 h. A constant depth would indicate death, with the carcass floating or sitting on the seafloor. Upon release from a fish and surfacing, each tag would transmit data to the Wildlife Computers Inc. portal through Argos system satellites (Argos Services, Ramonville-Saint-Agne, France). The sPAT has sensors that record daily minimum and maximum values for depth and temperature and the daily change between minimum and maximum light levels. Depth information from the final 5 d prior to pop off of each tag was recorded at 10-min intervals in addition to the daily minimum and maximum depths.

Swordfish were tagged aboard pelagic longline vessels of the U.S. commercial fishery, off the coast of South Carolina in the Charleston Bump region, off northeastern Florida in the vicinity of the Blake Escarpment, and off northeastern New Jersey around the Hudson Canyon (Fig. 1). Fishing took place between January 2021 and October 2023, when vessels were available. These vessels targeted albacore (*Thunnus alalunga*) and bigeye tuna using gangions with lines of approximately 12–20 m and 16/0 non-offset circle hooks baited with dead squid (*Illex* spp.), ballyhoo (*Hemiramphus brasiliensis*), or Atlantic mackerel (*Scomber scombrus*). The pelagic longline was deployed normally, with gear recovery starting after soak times of 8–12 h.

¹ Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.



Size was visually assessed as straight LJFL, the straight-line distance between the tip of the lower jaw and the fork of the tail. Any swordfish between 74 and 119 cm LJFL (up to a visually estimated 120 cm) that was actively capable of swimming and had not sustained any mortal wounds (e.g., missing a caudal fin or significant chunks of the body) was considered a candidate for tagging. Smaller individuals (<74 cm LJFL) were not tagged because of concerns regarding the effect of tag size and the associated drag on and potential damage to fish from a tag (Grusha and Patterson, 2005), and larger individuals (>120 cm LJFL) could not be tagged because they were retained by crew for market. Size was estimated visually without removing fish from the water, although in instances when the size was close to either end of the size bracket, the fish was removed, measured, and tagged as quickly as possible. Tags were attached through the dorsal musculature such that the anchor would lock behind the pterygiophores below the dorsal fin midpoint. Tagging was conducted as

quickly as possible, and the time taken never exceeding 1 min. Hooks were removed from individuals that were easily accessible, or the leader was cut as close to the hook as possible.

Survival of individuals was inferred by using the change in depth as well as changes in recorded environmental data (temperature and ambient light). Horizontal net displacement (HND) was also analyzed to support survival estimates and determine habitat utilization. Depth profiles were the primary means of determining mortality, and individuals whose movements included regular changes in depth over the course of the 30-d deployment period were considered to have survived their tagging. Depth time series were compared with those from Fenton (2012) and Lerner et al. (2013) to determine if behavior was normal (occupying the mesopelagic zone during the day and coming near the surface during the night) and to identify anomalies resulting from tag ingestion by a predator, such as a constant temperature despite increasing

depth (e.g., Kerstetter et al., 2004). Both null movement (no depth changes within 24 h) and a steady increase in depth up to the maximum resulting in pop off of a tag were inferred as mortalities. The HND was calculated as the straight-line distance between the deployment location and the tag's first transmission to the Argos satellite system by using QGIS, vers. 3.30.2 (QGIS Development Team, 2023). Habitat preference was differentiated from mortality for short distances if the depth and environmental data did not indicate predation and the tag remained on the fish for the full 30-d period. A Kruskal–Wallis one-way analysis of variance was conducted to determine if there was a significant correlation ($P \leq 0.05$) between distance and direction traveled.

To analyze how the small sample size could have influenced the results of this study, a bootstrapping simulation was conducted by using Release Mortality (vers. 1.1.0), software developed by Goodyear (2002). This program was used to estimate the 90% confidence intervals of postrelease mortality over 10,000 simulations with a series of specified process, experimental, and decision variables. Initially, values of all variables except the release mortality fraction were set to zero, assuming no error sources (e.g., tag shedding or tags that did not transmit data). Then, 2 more simulations were conducted to analyze how outside errors may have influenced results. For these additional simulations, the variables were kept the same except for the decision variable *tags not reporting*. In the first simulation, tags that did not transmit data were included, and in the second simulation, such tags were excluded. Values for all process and experimental variables were based on results of this study or from prior research and were as follows: *tagging mortality fraction* (0.00; i.e., no assumption of tag-induced mortality), *tag failure probability* (0.25), *tag shedding probability* (0.04), and *natural mortality* (0.2, based on Griggs et al., 2005). The programmed duration from tagging to tag pop off was set to 30 d for all simulations, and the number of tags was set to 20 and doubled with every iteration until the confidence intervals were within 5% of the assumed true value. This procedure was similar to those used in prior studies (Goodyear, 2002; Fenton, 2012; and Rewis, 2023). For decision variables, data from tags reporting normally were included as data for surviving swordfish.

Maximum depth and time intervals taken from the final time series were qualitatively used in analysis of vertical habitat utilization for the 5 d prior to sPAT release to confirm individual swordfish survival. The time series from tags deployed on surviving fish were graphed to show depth variations over this interval. To avoid including in analysis of habitat utilization any atypical behaviors that may result from the capture and tagging of swordfish, only data recorded after 10 d was used (Hoolihan et al., 2015). Depth profiles from all tags were also evaluated for daily vertical movements, and a combined daily behavior trend line was created by using a generalized additive model. Statistical analyses in this study were conducted in RStudio, vers. 2023.03.0 (Posit Software PBC, Boston, MA). Daytime and nighttime intervals were determined

by inputting the first location of tags after pop off into the web-based NOAA Solar Calculator (NOAA Earth System Research Laboratories, Global Monitoring Laboratory, available from website).

Results

Data from 16 of the 24 tags deployed (Table 1) were used for analysis of mortality, with 9 tagged fish surviving for the 30-d recording period. Five tags transmitted no data or began recording prior to deployment and did not provide relevant data. Additionally, 2 tags did not provide data, and 1 tag was shed. Habitat utilization was analyzed for the 9 fish that did not die. However, data from one of these tags was excluded from HND analysis because of an inaccurate pop-off location (tag 199973), and data from 2 tags indicate that fish experienced depths considered outlier values (tags 199966 and 199973). Multiple data points in the time series from tag 199966 indicate that the fish stayed at or near the surface for hours after sunrise. Although this trend was recorded only for this individual, it could be evidence of “basking” behavior that has been described previously (Carey and Robinson, 1981; Abascal et al., 2010; Fenton, 2012). Data from the last 4 d of the time series from tag 199973 indicate abnormal behavior, with the fish staying during night at increasingly deeper depths that were below the depths recorded by tags on other individuals.

Over the 30-d deployment period, 9 (56.2%) of the 16 fish for which data were analyzed survived. Between surviving individuals, HND varied from 22 to 783 km (mean: 367 km). The minimum average daily swimming speed varied between 1 and 26 km/d (mean: 12 km/d). Size of individual fish did not significantly influence the distance traveled ($df=3$, $P=0.85$). The orientation of HNDs was generally parallel to the coastline, with 6 of the tagged fish traveling northward and 2 tagged fish moving southward (Fig. 1). The 2 individuals that moved southward experienced the shortest HNDs, traveling only 22 and 116 km, whereas the northward moving fish all traveled a minimum distance of 168 km. There was a significant difference between the directional movement and distance traveled ($df=1$, $P=0.05$). Tagged swordfish inhabited the mesopelagic zone during the day and the epipelagic zone at night (Suppl. Fig. 1) (online only). The range of daytime depths was approximately 350–900 m, with an average of 544 m (standard deviation 154). Swordfish began to ascend approximately 2.6 h before dusk, then would begin to descend back to mesopelagic depths about 1.2 h before dawn. A generalized additive model of the daily movements from 7 of the surviving swordfish was constructed, explaining 74.5% of the variation in occupied depth (Suppl. Fig. 1) (online only).

Of the 7 tags on swordfish that died, 5 tags sank to the seafloor within hours of tagging, 1 tag remained at the surface with the fish making a brief dive midway through the second day before returning to the surface, and 1 tag recorded abnormal movement for multiple days before

Table 1

Summary of movement and mortality assessments, based on data from 24 survivorship pop-up archival tags, for undersized swordfish (*Xiphias gladius*) released from pelagic longline gear deployed in the western North Atlantic Ocean during January 2021–October 2023. Horizontal net displacement (HND) was recorded to support survival estimates and determine habitat utilization. Mortality categories include alive, tag not reporting (NR), dead, premature release (PMR), and not determined from available data (ND). An asterisk (*) indicates that the length is an actual measurement of an individual removed from water, rather than a visual estimate.

Tag ID	Date tagged	Length (cm)	HND (km)	Days at liberty	Mortality
199947	2021-05-30	110	770.74	30	Alive
199948	2021-06-03	120	–	–	NR
199950	2021-06-03	90	120.58	1	Dead
199951	2021-05-30	110	169.61	30	Alive
199952	2021-06-03	115	–	–	NR
199954	2021-05-30	120	363.76	30	Alive
199955	2021-05-30	100	116.00	30	Alive
199956	2021-05-31	100	140.82	1	Dead
199957	2021-05-30	110	167.82	30	Alive
199958	2021-06-02	100	119.24	1	Dead
199959	2021-05-30	122	1690.45	30	ND/PMR
199960	2021-01-21	118*	11.68	3	Dead
199961	2021-05-31	115	–	22	ND
199962	2021-05-31	100	–	–	NR
199963	2021-06-03	90	–	–	NR
199966	2021-06-02	90	543.00	30	Alive
199967	2021-01-30	119*	193.25	5	ND/PMR
199973	2021-06-02	90	110.62	30	Alive
199975	2021-06-05	80	134.77	1	Dead
199986	2021-05-30	100	170.14	1	Dead
199987	2021-01-31	110*	33.66	1	Dead
199989	2021-05-30	100	22.19	30	Alive
199993	2023-10-08	80*	2000.00	30	ND
199994	2023-05-30	100	783.38	30	Alive

ceasing to capture any sort of intentional vertical movement indicative of a swimming fish and floating to the surface. All recovered depth profiles from tags are provided in Supplementary Figure 2 (online only).

The results of the analyses done with the Release Mortality software indicate that some 1200 tags would be needed to verify the precision of the mortality estimate to 5% above or below the confidence interval, with a true mortality rate of 43.8% (90% confidence interval: 29.2–58.3%) (Suppl. Fig. 3) (online only). The addition of variables, such as *tag failure probability*, *tag shedding probability*, and *natural mortality*, led to a widened 90% confidence interval of 27.8–66.7%.

Discussion

The expense of satellite archival tags and their deployment continues to present barriers to collecting data from fish tags in numbers large enough to allow rigorous

analysis across multiple condition strata, such as accumulated hooking time. One of the many challenges faced during this study was that the concentrations of undersized swordfish and other bycatch in the catch of cooperating vessels could not be known in advance, making it difficult to reliably tag undersized individuals on any given trip. The tags deployed in this study were few in number. However, results of the bootstrapping analyses indicate that the number of deployments needed for more precise confidence intervals are not practical because of both the cost of tags and the logistical effort necessary.

Adding to the difficulty of obtaining significant sample sizes is the potential for tags to fail to report usable data. Scientists encounter several challenges in deployment of electronic tags on pelagic fish species, and tags not reporting data is a common occurrence in studies involving satellite tags on swordfish (Canese et al., 2008). Tags can fail to report for several reasons, including biofouling (accumulation of aquatic organisms) that can prevent a tag from surfacing and transmitting data, battery

drain over extended periods prior to deployment (Hays et al., 2021), entanglement with debris (Fenton, 2012), and damage due to predation (Kerstetter et al., 2004). In addition to tags that do not report data, the premature release of tags due to detachment either of the anchor or a break of the wire between the tag and the anchor, tag shedding, can also reduce sample sizes even though tags that are released early do report some data. Tag shedding is common in studies of istiophorid billfishes (e.g., Canese et al., 2008). One tag (tag 199967) was deemed to have prematurely shed on the basis of the normal behavior of the fish prior to tag release. Because the fate of the fish after tag 199967 was released is unknown, mortality cannot be determined, even if the behavior inferred from the data is that of a healthy individual.

It is likely that mortalities observed in this study were related to exposure to stressors incurred over the extended pelagic longline soak times, given the short time span between release and apparent mortality events. However, it is not practical to attach electronic timers that record the duration of hooking across the hundreds of hooks deployed in a standard pelagic longline set. Mortality occurred within hours for all but one fish, which died 3 d after tagging, appearing to be unable to stay at depth and not following the movement patterns typical for swordfish. Pelagic longline gear likely subjects swordfish to greater post capture stress than other gear types, because of the higher potential for swordfish to be on the line for extended periods due to the length of time that longlines need to be deployed. Indeed, previous research indicates that at-vessel mortality of undersized swordfish within the U.S. Atlantic fleet has been 65–79% since the implementation in 2004 of the requirement for the use of circle hooks (ICCAT²). High mortality rates have been reported throughout the Atlantic Ocean for other pelagic longline fleets, such as the fleet that operates in Portugal and has a reported at-vessel mortality of 87.8% (Coelho and Muñoz-Lechuga, 2019). Mortality is influenced by several factors, including vessel practices, seasonality, and hooking location and therefore varies temporally and between fleets. For example, Serafy et al. (2012) found that the required adoption of circle hooks in U.S. pelagic longline and recreational tournament fisheries has led to an 8% reduction in mortality for billfishes and may explain why these U.S. fisheries have lower mortality rates than other fleets that deploy J hooks.

The survival rates of undersized swordfish caught on pelagic longline gear is lower than that reported for other gear types. Tracey et al. (2023) tagged swordfish exclusively caught on rod-and-reel gear with angling times typically of only around 1 h and reported a postrelease survival rate of 44% (when including moribund individuals deemed not suitable for tagging). Fenton (2012) reported survival

rates of 64.3% for swordfish caught on buoy and recreational rod-and-reel gears and reported no at-vessel mortality prior to tagging. However, at-vessel mortality might be expected to be lower given that these gear types are deployed for shorter durations than pelagic longlines and are more closely monitored by vessels. As such, individual fish do not spend as much time on these other gear types compared to pelagic longlines, which soak in the water for 10–14 h (NMFS, 2022) and then take additional hours to fully retrieve. The results of our study also indicate that swordfish have lower postrelease survival on pelagic longlines than istiophorid billfishes, whose survival typically is around 86% on this gear type (reviewed by Musyl et al., 2015). Although the reason for this difference in survival is not clear, it is possible that the biology of swordfish differs from that of istiophorids such that the species is less capable of recovering from catch-induced stress.

Within the context of commercial pelagic longline fisheries, most undersized swordfish do not survive capture events. Given the 65–79% (ICCAT²) at-vessel mortality of undersized swordfish in U.S. fisheries and the estimated 43.8% postrelease mortality determined in our study, the total mortality for undersized swordfish would likely fall between 80% and 88%. Such a result calls into question the overall effectiveness of the current minimum size required for retention and its effect on fish biomass and stock conservation. To put this range of total mortality into perspective, consider that, of the 5844 undersized swordfish caught per year on average between 2017 and 2021 (NMFS, 2022), 4675–5143 swordfish too small to retain likely died in that period within the U.S. fishery alone. Although this amount is small in comparison to actual retained catches (Fig. 2), it represents a substantial level of mortality of unharvested fish.

Regulations that mandate the discard of all individuals that do not meet the minimum size for retention have discouraged the targeting of small swordfish, given that the reported percentage of landed swordfish that were undersized between 2000 and 2015 internationally was reduced from 33% to 23% (ICCAT, 2019). Revisiting the International Commission for the Conservation of Atlantic Tunas management rule that was used from 1990 to 2004, an alternative of a larger minimum size with retention of a percentage of undersized swordfish, could reduce the number of dead swordfish discards without incurring additional mortality in the stock. However, such a measure would need to be approached with caution. An overcorrection could undo decades worth of stock rebuilding and incur opposition from other domestic swordfish fishery stakeholders. The results of this study do not provide clear support for any proposed management policy changes.

If, after further research, the requirement of a minimum size for retention is deemed to insufficiently reduce stock mortality, additional policies could be implemented in U.S. fisheries to further reduce catch rates of undersized swordfish or to improve their survivorship. The mandate for the use of circle hooks in the U.S. pelagic longline fleet has led to an 8% reduction in mortality within swordfish fisheries (Serafy et al., 2012), but many international fleets resist

² ICCAT (International Commission for the Conservation of Atlantic Tunas). 2022. Report of the 2022 ICCAT Atlantic swordfish stock assessment (online, 20–28 June 2022), 96 p. ICCAT, Madrid, Spain. [Report on stock assessment meeting held by ICCAT.] [Available from website.]

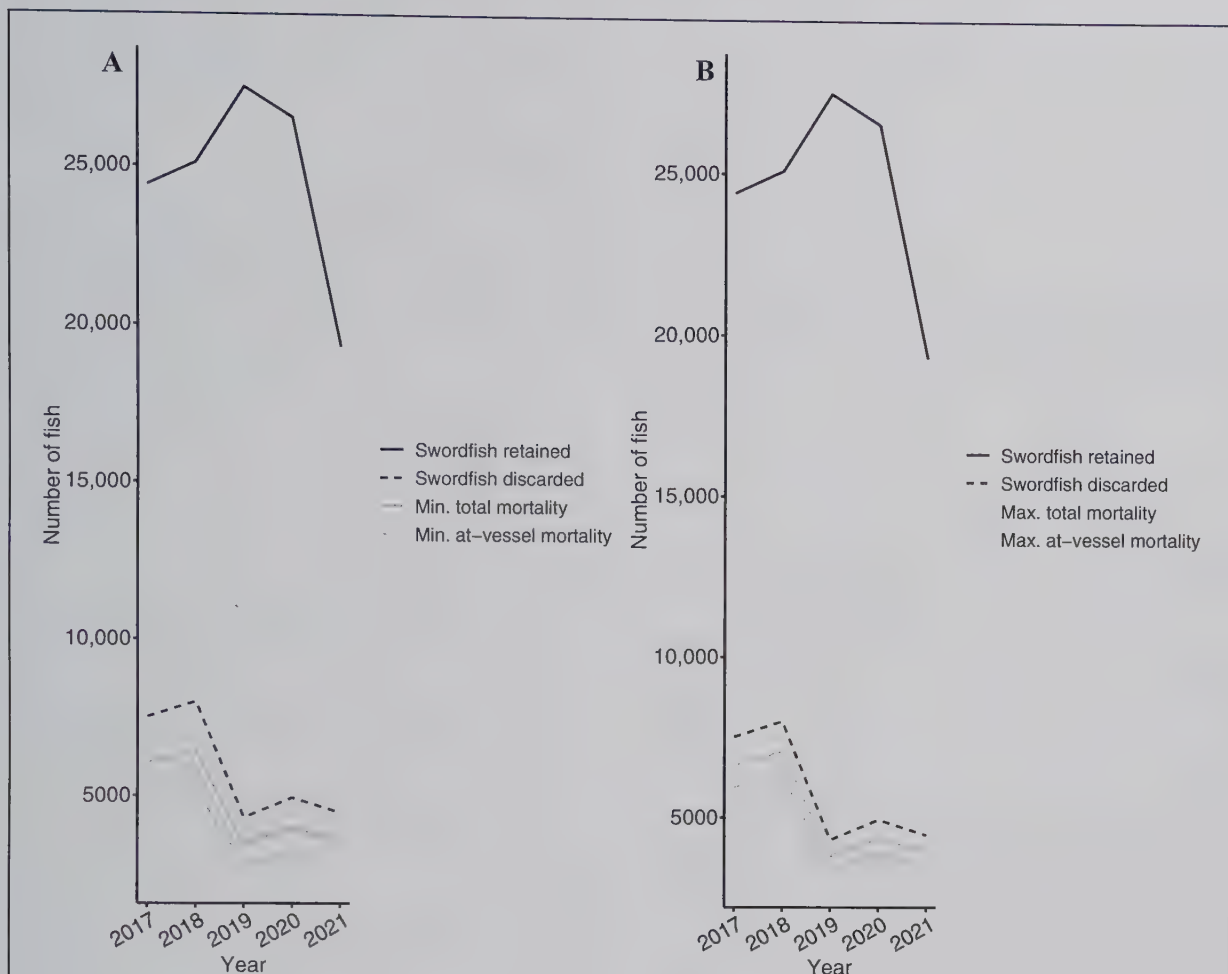


Figure 2

Estimated numbers of swordfish (*Xiphias gladius*) retained and discarded in the U.S. pelagic longline fishery during 2017–2021, based on the assumption of (A) minimum (65%) and (B) maximum (79%) estimates of at-vessel mortality from the stock assessment meeting held in 2022 by the International Commission for the Conservation of Atlantic Tunas. Total mortality represents the combination of at-vessel mortality and the 43.8% postrelease mortality found in this study. Data for captured swordfish that were discarded because they were smaller than the size required for retention (119 cm in lower jaw fork length) and that were retained in the fishery are from the stock assessment and fishery evaluation report for Atlantic highly migratory species published by the National Marine Fisheries Service in 2022. The difference between panels in values shown is the addition of the numbers of retained swordfish in panel B.

regulation regarding the use of circle hooks. Limiting soak times could reduce the physiological stress on caught fish and decrease mortality, but it also could likely lead to a reduction in target species yield, which would result in an increase in fishing effort to maintain current catch levels. An increase in hook size could reduce interactions with undersized swordfish (Kerstetter³) but may also reduce catch of smaller target species, such as bigeye tuna and yellowfin tuna. A combination of measures to reduce the

mortality of undersized swordfish should continue to be examined.

The study described herein is the first assessment of the postrelease survival rate of undersized swordfish caught on pelagic longline gear, information that could be used to provide a correction factor for future stock assessments. Although the sample size was small, the postrelease survival rate of 56.2% is both lower than that for several istiophorid billfishes released from pelagic longline gear in U.S. fisheries and lower than that for swordfish released from buoy gear or recreational rod-and-reel gear. Future research should focus on increasing the number of tags deployed to refine this preliminary estimate of survival, and this work should include analysis of variables such as temperature, time hooked on gear, and hook type, as well

³ Kerstetter, D. W. 2004. Preliminary gear comparison between 16/0 and 18/0 sizes non-offset circle hooks in the Southeastern U.S. coastal pelagic longline directed swordfish fishery. VIMS Mar. Resource Rep. 2004-07, 19 p. Va. Inst. Mar. Sci., Coll. William Mary, Gloucester Point, VA. [Available from website.]

as associated physiological stress responses. Although a majority of tagged individuals survived release in this study, this result combined with the high at-vessel mortality rates estimated in previous studies for swordfish caught by pelagic longline gear indicates that minimum retention lengths may not have substantial conservation benefits beyond discouraging targeting. Management policies could be altered without biological detriment to the stock to allow some incidental retention of undersized swordfish while also discouraging the targeting of undersized individuals, but such options are very likely to be controversial among current stakeholders of the swordfish fishery.

Resumen

Se colocaron marcas archivadoras desplegables de supervivencia (sPAT) a peces espada (*Xiphias gladius*) liberados en palangreros pelágicos comerciales estadounidenses en el océano Atlántico Norte occidental. El propósito era determinar la supervivencia después de la liberación de individuos de talla inferior a la talla mínima de retención (119 cm de longitud mandíbula inferior-furcal) establecida por la Comisión Internacional para la Conservación del Atún Atlántico (CICAA). Entre 2021 y 2023, se colocaron 24 sPAT con una duración programada de 30 días para medir la profundidad, la temperatura y la luz ambiental. La temperatura y la profundidad se registraron como mínimos y máximos diarios, y el nivel de luz se registró como un cambio diario entre los valores mínimos y máximos. Los últimos 5 días de datos de profundidad también se registraron a intervalos de 10 minutos antes de la liberación para evaluar los movimientos a escala fina coherentes con la supervivencia. Diecinueve marcas transmitieron datos, y 16 de ellas tenían datos suficientes para el análisis. De estas marcas, 9 sPAT (56.2%) transmitieron datos consistentes con los movimientos de un pez espada superviviente. Esta tasa de supervivencia es inferior a las registradas en estudios anteriores utilizando caña con carrete y boyas para pez espada, lo que indica que la captura con palangre pelágico causa un mayor estrés al pez espada que esos otros equipos de pesca. Este resultado, combinados con estudios previos sobre las tasas de mortalidad a bordo, indica que la norma actual de CICAA sobre la talla mínima de retención ofrece beneficios limitados para la conservación de las poblaciones del pez espada.

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Abstract—The abundance of black sea bass (*Centropomus striata*) has increased in coastal waters of the north-eastern United States, and the implications of that change for regional food webs remain poorly understood. We used DNA metabarcoding of 2 gene regions, the mitochondrial cytochrome *c* oxidase subunit I (COI) region and the V9 hyper-variable region of 18S rRNA, to classify prey in gut contents of juvenile black sea bass collected from a nearshore habitat in Long Island Sound (LIS). Using sequence numbers for the COI and V9 regions, we found that gut contents were dominated by the crustacean superorder Eucarida, followed by 2 polychaete species, and an invasive amphipod, *Grandidierella japonica*. Bivalves, gastropods, and ostracods were less abundant. Fish prey, including northern pipefish (*Syngnathus fuscus*) and Atlantic silverside (*Menidia menidia*), were present (>10% of sequences) in the stomachs of 2 large black sea bass. High variability in sequence numbers was evident between individuals for both the COI and V9 gene regions. Taxonomic composition of prey items also differed: eucarids contributed >87% of V9 gene sequences in samples from 51 stomachs, amphipods composed >50% of sequences in samples from guts of 21 fish; and polychaetes dominated with >75% sequences in samples from 6 stomachs. We concluded that, during settlement pulses, juvenile black sea bass may compete with other benthic predators for decapod, polychaete, and amphipod prey in LIS nearshore habitats.

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Using DNA metabarcoding to reveal prey diversity in diets of juvenile black sea bass (*Centropomus striata*) in Long Island Sound in the Northwest Atlantic Ocean

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Human intervention, exploitation, and climate change have increasingly led to rapid ecosystem and regime shifts worldwide (Perry et al., 2005; Poloczanska et al., 2013). Long Island Sound (LIS), a tidal estuary of the Northwest Atlantic Ocean located at 41°19'24"N, 72°0'55"W, borders one of the most densely populated metropolitan areas in the world and consequently is part of one of the most anthropogenically affected coastal regions in North America. The waters of LIS have been warming at a rate about 4 times faster than the global average over recent decades (Snyder et al., 2019), causing a rapid, ongoing reorganization of the LIS ecosystem evidenced by a shift from cold-water adapted to warm-water adapted marine communities (Howell and Auster, 2012; Crosby et al., 2018; Snyder et al., 2019). Regime shifts are often associated with rapid increases in the abundance of new or previously rare species, which then assume new, self-reinforcing trophic roles (Alheit et al., 2005). In LIS, this type of response to a regime shift is a likely explanation for the increase in abundance of black sea bass (*Centropomus striata*), an omnivorous mesopredator (a predator that

occupies a mid-ranking trophic level in a food web) of the grouper family (Serranidae), in waters on the continental shelf of the Northwest Atlantic Ocean (Bell et al., 2015; McMahan et al., 2020). This species has become more abundant while populations of cold-water species have continued to decline in abundance (Collie et al., 2008; Howell et al., 2016; Frisk et al., 2018). Indeed, abundance of black sea bass in LIS has risen by one order of magnitude since 2010 (Zavell et al., 2024).

The large increase in abundance of black sea bass in LIS could be affecting the food web and productivity in profound ways, but little is currently known about diets of black sea bass in the region (Taylor et al., 2023). Results from previous diet studies of black sea bass captured south of the Hudson Canyon, an extension of the Hudson River Valley located about 160 km south-east of New York City, indicate a wide, generalist diet, including crustacean, mollusk (especially squid), and fish species (Sedberry, 1988; Hood et al., 1994; Steimle et al., 1999). However, the diet of black sea bass north of the Hudson Canyon is less known, particularly for

juveniles, which now arrive during July–September in large numbers to settle in shallow nursery habitats in LIS. It is in these LIS nursery habitats that settling black sea bass could first affect local food webs by potentially outcompeting other benthic predators for prey. No data are currently available on the diets of juvenile black sea bass in LIS, and this knowledge gap limits projections of possible ecosystem effects.

Research on fish diets has traditionally relied on morphological inspection of preserved gut contents, requiring diverse taxonomic expertise and a considerable time commitment of research staff (e.g., Taylor et al., 2023). Moreover, morphological approaches detect large, hard-bodied prey like fish more reliably than soft-bodied, small, and less abundant prey items like gelatinous zooplankton (Amundsen and Sánchez-Hernández, 2019). The use of DNA metabarcoding has provided new insights into marine biodiversity (Bucklin et al., 2016; Govender et al., 2023). Metabarcoding allows identification of taxa from a sample based on analysis of short regions of DNA sequences called *barcodes* (Taberlet et al., 2012). One commonly used barcode is a portion of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene region (Bucklin et al., 2021); additional markers include hypervariable regions in rRNA, including the V9 hypervariable region of 18S rRNA (Amaral-Zettler et al., 2009). Metabarcoding relies on high-quality databases of reference gene sequences to identify species or taxonomic groups in a sample (Coissac et al., 2012; Bucklin et al., 2021; O'Brien et al., 2024).

Metabarcoding holds promise for fish diet studies because DNA samples can be processed in a relatively short amount of time and highly digestible, soft-bodied prey items that may elude morphological analyses can be detected (Schroeder et al., 2020). The diets of yellowfin seabream (*Acanthopagrus latus*) (Su et al., 2018; Pan et al., 2021) and Arctic lamprey (*Lethenteron camtschaticum*) (Shink et al., 2019) have been successfully examined by using metabarcoding. The utility of metabarcoding with the V9 region of 18S rRNA has been previously demonstrated in an examination of diets of 2 zooplanktivorous clupeids: European pilchard (*Sardina pilchardus*) and European sprat (*Sprattus sprattus*) (Albaina et al., 2016).

In this study, we used metabarcoding to examine the diet of juvenile black sea bass collected from a shallow, coastal habitat in LIS. We used the COI and V9 barcodes to characterize the prey spectrum and identify dominant hard- and soft-bodied prey taxa that could be affected by the expansion of the range of black sea bass in coastal regions of the Northwest Atlantic Ocean. We also tested the hypotheses that the use of the 2 gene regions for metabarcoding would yield qualitatively consistent results and that diet composition would vary with fish size and between 3 sampling dates in August 2020.

Materials and methods

Sample collection and DNA extraction

Juvenile black sea bass (≤ 14 cm in total length [TL]; Miller et al., 2016) were collected by using beach seine

nets (30 × 2 m) in Mumford Cove, a small, seagrass-dominated embayment off Connecticut in the eastern area of LIS. On each of 3 sampling dates, 6, 14, and 26 August 2020, collections were composed of up to 35 juveniles of similar lengths, with ranges of 22–51 mm TL, 29–62 mm TL, 31–69 mm TL, respectively, and an overall mean of 38 mm TL (standard deviation 7). Four large juveniles (>135 mm TL) were also collected for comparison of diets with those of smaller juveniles. The fish were removed from the seine net and immediately flash frozen in liquid nitrogen. A total of 107 specimens were analyzed in this study. In the laboratory, fish were partially thawed to dissect stomach contents but kept on ice to ensure stability of DNA. The stomach lining was removed, and gut contents were homogenized in buffer solution of sodium dodecyl sulfate and digested in a 55°C water bath for 6–7 h. DNA was extracted by using phenol-chloroform-isoamyl alcohol (mixture ratio: 25:24:1) and then a DNeasy PowerClean Cleanup Kit¹ (Qiagen, Hilden, Germany).

Amplicon generation and sequencing

Purified DNA was amplified for the target COI and V9 regions through polymerase chain reaction (PCR). MiSeq adapters (Illumina Inc., San Diego, CA) were added to all primers used for amplification. A 313-base-pair (bp) region of the COI gene sequence was amplified with Invitrogen Platinum *Taq* DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA) reagents and 20 ng of DNA by using PCR primers, mlCOIintF and jgHCO2198, and following published protocols (Geller et al., 2013; Leray et al., 2013). For the amplification of the V9 gene region, 10 ng of DNA from each individual stomach was mixed with KAPA HiFi PCR Kit (Roche Diagnostics Corp., Indianapolis, IN) and 1 μ L of each primer (10 μ M), 1510R and 1380F, by using published protocols (Amaral-Zettler et al., 2009). The sample products from amplification of both the V9 and COI regions were examined by using electrophoresis in a 2% gel with a 50-bp marker.

Library preparation entailed adding Nextera XT index primers (Illumina Inc.) for a second PCR cycle. Successful library attachment was verified by using a High Sensitivity D1000 ScreenTape Assay for 4200 TapeStation Systems (Agilent Technologies Inc., Santa Clara, CA). Bidirectional sequencing of libraries was carried out at the University of Connecticut Center for Genomic Innovation by using a MiSeq System sequencer and the MiSeq Reagent Nano Kit v2 (Illumina Inc.) spiked with a minimum of 20% PhiX Sequencing Control v2 (Illumina Inc.) to account for amplicon sequence redundancy.

Bioinformatics

Gene sequences from 35 of 100 samples taken from stomachs of black sea bass were available for COI analysis.

¹ Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.

Paired-end reads for COI sequences were processed with a custom mothur script (vers. 1.44.3; Schloss et al., 2009), executed on the Xanadu computing cluster (Xanadu Quantum Technologies, Toronto, Canada) at the University of Connecticut. Sequences were filtered by using length and sequence quality control values. Generated contiguous sequences (>150 bp) were aligned to sequences in the global MetaZooGene Atlas and Database (O'Brien et al., 2024; database available from website). The PCR errors and chimeras were filtered out by using Unoise and Vsearch in mothur (Rognes et al., 2016). Taxonomic classification and species identification were based on sequences and operational taxonomic units for the COI region by using a naive Bayesian classifier algorithm in mothur against the data set for the North Atlantic Ocean in the MetaZooGene Atlas and Database (Bucklin et al., 2021). Species-level taxonomic identification was carried out by using >97% bootstrap values after 100 iterations. Bootstrap values in COI metabarcoding analyses usually range from 95% to 100% to maximize recovered genetic diversity while controlling for sequence errors (Porter and Hajibabaei, 2020).

Sequences from 99 of 100 samples taken from stomachs of black sea bass were available for analysis of the V9 region. The reads of the V9 gene sequences were also analyzed with the tailored mothur pipeline script (Schloss et al., 2009) and executed on the Computational Biology Core at the University of Connecticut. Contiguous sequences were assembled from forward and reverse MiSeq reads. Contiguous sequences containing ambiguous base calls and quality Phred scores <30 were discarded. To mitigate any PCR biases, we screened for and removed chimeras using Vsearch in mothur (Rognes et al., 2016). Remaining sequences were aligned to the sequences in the SILVA reference database, release 132 (Quast et al., 2012). Aligned sequences that did not span the entire V9 region were removed to improve the accuracy of the generated operational taxonomic units. To further address potential PCR bias, we used the Unoise 37 method (Edgar²) in mothur to impose a 2-bp difference limit between sequences before clustering of operational taxonomic units. Taxonomic assignment with identification bootstrap values ≥80% after 100 iterations were carried out by using a Bayesian classifier (Wang et al., 2007) and a modified database developed from the data set of the small subunit (18S rRNA) sequences in the SILVA database, release 132 (Blanco-Bercial, 2020). Bootstrap values for the V9 region are lower than those for the COI region because of the slower rate of evolution. This difference in rate of evolution results in conserved regions, facilitating the design of primers and allowing taxonomic assignment to family level at best (Amaral-Zettler et al., 2009).

On the basis of the taxonomy summary files for COI generated in mothur (Wang et al., 2007), we focused on gene sequences for 6 taxonomic prey groups: Eucarida (i.e.,

Decapoda and Euphausiacea), Polychaeta, Amphipoda, Actinopterygii, Copepoda, and Gastropoda. For the V9 region, we additionally included sequences for Ostracoda, Bivalvia, Hemichordata, and Ascidiacea, while disregarding sequences for Actinopterygii because the V9 region cannot be used to distinguish between the tissues of fish prey and the stomach of the predator. Taxa represented by <100 sequences were removed from further analysis.

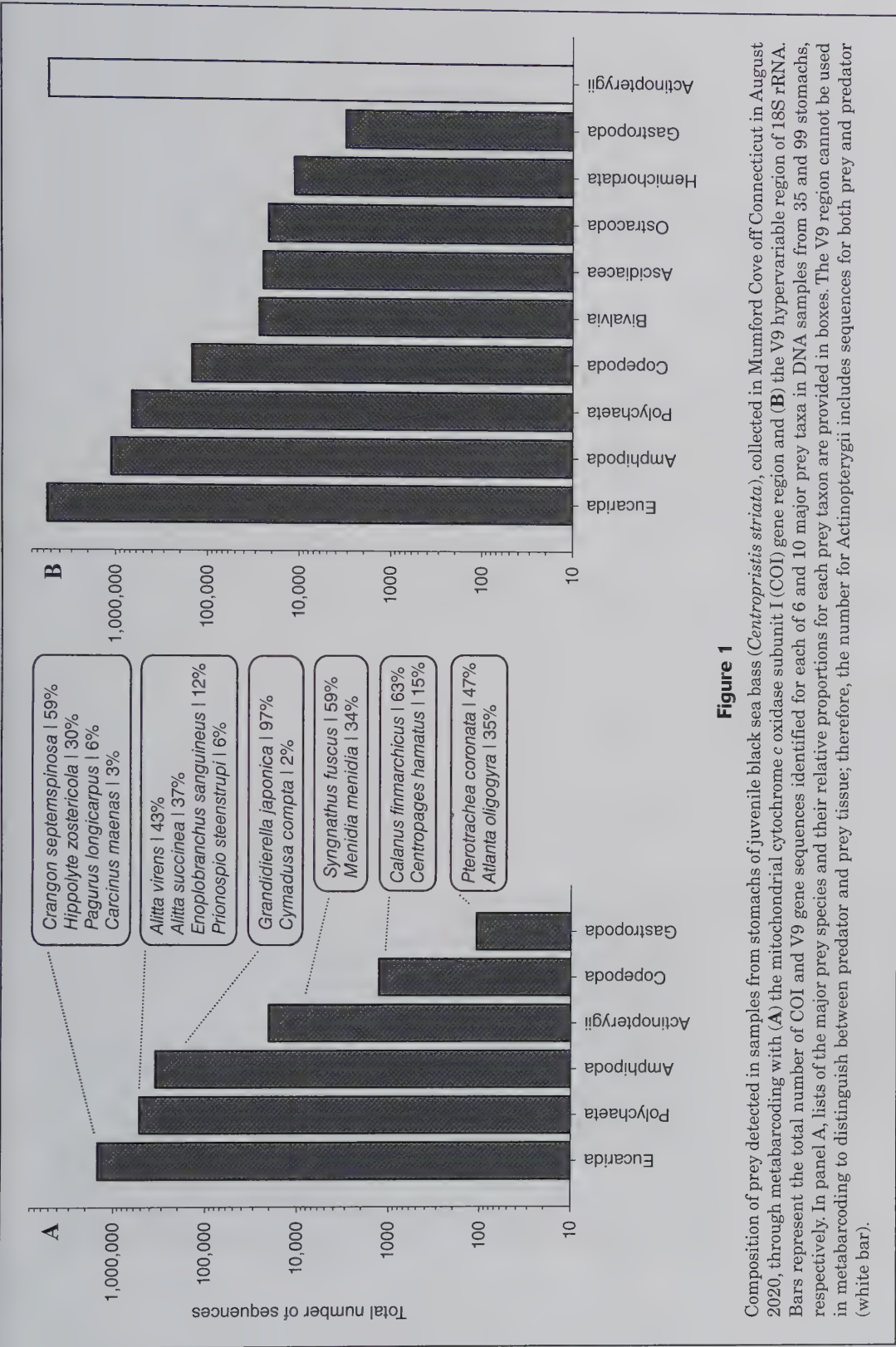
We used cumulative COI and V9 sequence numbers across all stomach samples to describe the overall prey composition of juvenile black sea bass. Variation between samples was examined by comparing taxon-specific sequence numbers expressed as proportions of the total sequence number for all samples. Kruskal–Wallis tests were used to compare log-transformed ($\log_{10}(N+1)$) V9 sequence numbers of Polychaeta, Amphipoda, and Copepoda between the main cohort sample (i.e., juveniles 22–69 mm TL) and the 4 individuals >135 mm TL. To determine whether prey composition changed between the 3 sampling dates in August 2020, we used log-transformed V9 sequence numbers of the 9 prey taxa for each individual as input for a principal component analysis. Components with an eigenvalue >1 were extracted, rotated component scores were used to test for significant differences between sampling dates with a multivariate general linear model, and Fisher's least significant difference post-hoc tests were conducted. To test for collinearity between V9 and COI sequence numbers, we restricted the data set to only those individuals for which data for both barcodes were available (sample size $[n]=35$) and used Pearson correlation and linear regression to compare the log-transformed sequence numbers within each major prey group. These analyses were done in SPSS Statistics, vers. 20.0 (IBM, Armonk, NY).

All metabarcoding sequence data and associated meta-data have been deposited in the GenBank Sequence Read Archive (National Center for Biotechnology Information, available from website) under BioProject PRJNA1101022. The GenBank accession numbers for COI gene sequences are SRR28750955–SRR28750989 and for V9 gene sequences are SRR28745280–SRR28745384.

Results

Through metabarcoding of the COI region, we detected a total of 2.79 million sequences across 35 samples of the stomach contents of black sea bass (Fig. 1A). Over half of all sequences belonged to the superorder Eucarida (1.47 million sequences), represented almost completely by 3 decapod crustacean species: the sevenspine bay shrimp (*Crangon septemspinosa*) (59%), zoster shrimp (*Hippolyte zostericola*) (30%), and longwrist hermit (*Pagurus longicarpus*) (6%). The class Polychaeta (0.52 million sequences) was the second-most abundant taxon, consisting largely of sand worms (*Alitta virens*) (43%) and clam worms (*A. succinea*) (37%). Amphipod sequences (0.35 million sequences) were attributable almost exclusively to *Grandidierella japonica* (97%). An additional 20,000 COI

² Edgar, R. C. 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. BioRxiv 081257. [Available from website.]



sequences of fish (Actinopterygii) were attributable to northern pipefish (*Syngnathus fuscus*) (59%) and Atlantic silverside (*Menidia menidia*) (34%), and COI sequences of about 1000 Copepoda and about 100 Gastropoda were identified across all stomach samples (Fig. 1A).

Metabarcoding of the V9 region returned a total of 12.6 million sequences for 99 stomach samples from juvenile black sea bass (Fig. 1B). This number included 5.1 million sequences for Actinopterygii that were not included in analysis because V9 gene sequences cannot be used to distinguish between fish prey tissue and predator stomach tissue. Consistent with results from COI metabarcoding, most V9 sequences for taxa that were not fish were attributable to Eucarida (5.45 million sequences), followed by Amphipoda (1.11 million sequences), Polychaeta (0.67 million sequences), and Copepoda (0.15 million sequences). Sequences from the V9 region attributed to Bivalvia, Ascidiacea, Ostracoda, and Hemichordata were one order of magnitude rarer (0.01–0.03 million sequences) than sequences attributable to the previously mentioned more common taxa, but they were more frequent than sequences attributed to Gastropoda (0.003 million sequences) (Fig. 1B).

Both the number and taxonomic composition of detected sequences varied widely between juvenile black sea bass

(Fig. 2). The total number of COI sequences ranged from 20 to 120,000 (mean: 66,000) per stomach sample, and the total number of V9 sequences ranged from 7000 to 416,000 (mean: 120,000). In analysis with V9 sequence data, Eucarida were detected in all stomach samples, and in half of those samples ($n=51$) Eucarida accounted for >87% of all V9 sequences. Amphipoda and Polychaeta sequences were also detected in all samples, representing an average of 20% and 10% of sequences, respectively. In addition, results of gut content analysis indicate that 21 samples had >50% of total sequences classified as Amphipoda and that another 6 samples had >75% of total sequences classified as Polychaeta. Low numbers of Actinopterygii sequences were detected by using the COI region in 32 of 35 stomachs from black sea bass, but only the 2 largest individuals (138 and 145 mm TL; Fig. 2) had notable amounts of fish in their stomachs (i.e., >10% of all the sequences detected in each stomach). Shifts in the diet of black sea bass with size are also indicated by the V9 sequence data: comparisons with Kruskal–Wallis tests revealed that the 4 largest specimens had significantly lower average sequence numbers of Polychaeta ($P<0.001$), Amphipoda ($P=0.001$), and Copepoda ($P=0.002$) than smaller specimens (Fig. 3).

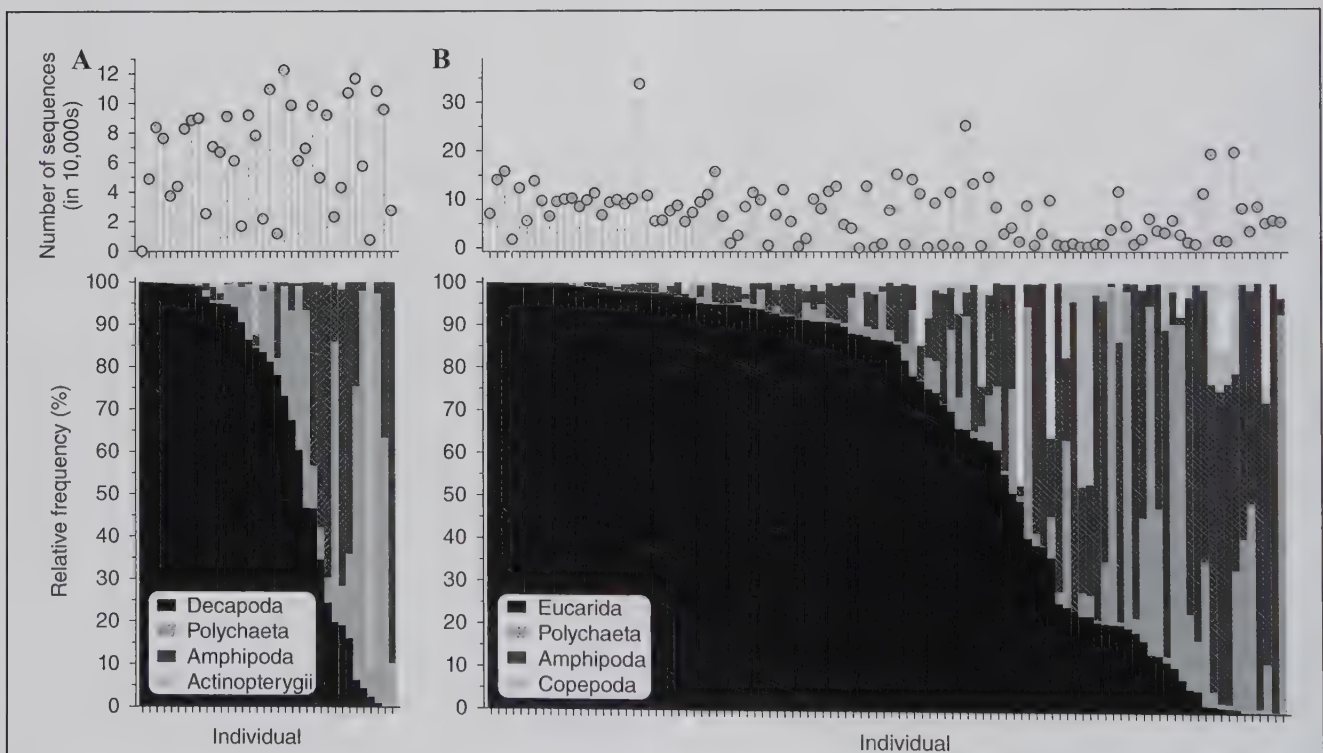
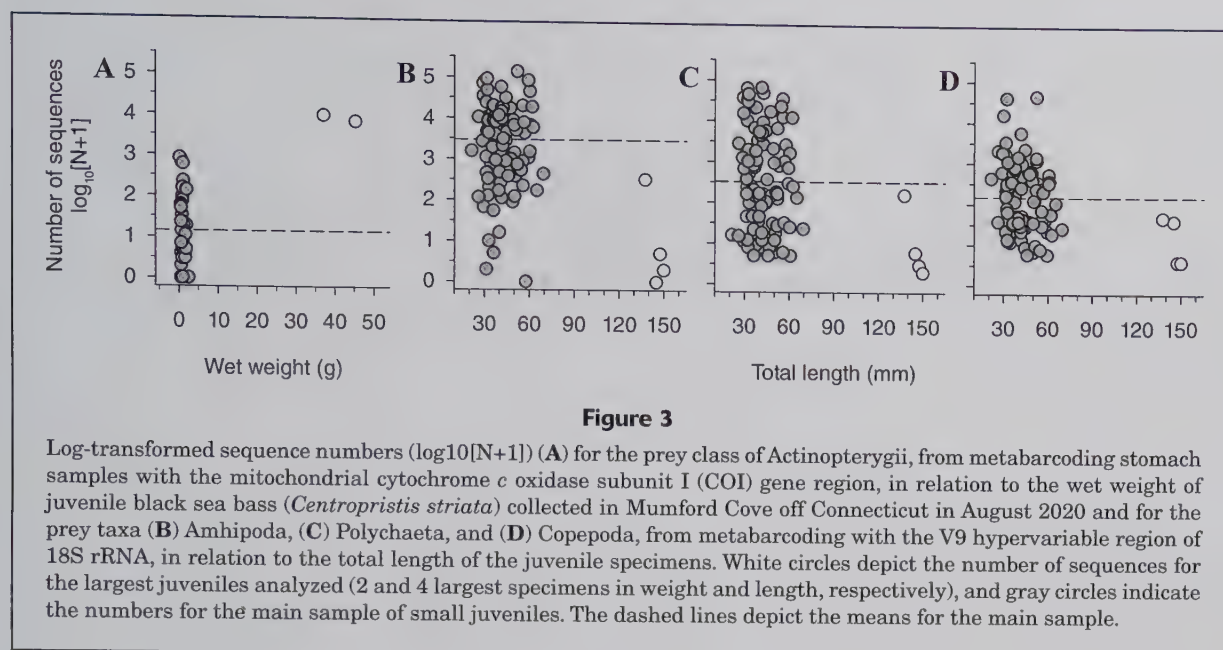


Figure 2

Results from analysis of stomach contents of juvenile black sea bass (*Centropristis striata*) collected in Mumford Cove off Connecticut in August 2020, through metabarcoding with (A) the mitochondrial cytochrome *c* oxidase subunit I gene region and (B) the V9 hypervariable region of 18S rRNA. In the top panels, data points indicate the variability between individuals in the number of sequences detected in stomach samples. In the bottom panels, bars indicate the relative frequencies of 4 major prey taxa found in stomach samples. Data for Actinopterygii are not reported because the V9 region cannot be used in metabarcoding to distinguish between predator and prey tissue.



In the principal component analysis of V9 sequence data for 9 prey taxa, 4 principal components (PCs) were identified that together explain 74% of the overall variability (PC1: 29%; PC2: 19%; PC3: 14%; PC4: 12%; Fig. 4). With the multivariate general linear model, significant differences between sampling dates were detected (Pillai's trace=0.55, $df=8$, $P<0.001$), and results from Fisher's least significant difference post-hoc tests indicate that PC1–3 all separated the first 2 collection dates (6 and 14 August) from the third date (26 August) ($P<0.035$), whereas PC4 separated 6 August from 14 and 26 August (all: $P<0.002$). Amphipoda and Polychaeta sequences were primarily responsible for distinguishing between samples collected on different dates along PC2. Gastropoda sequences separated sampling dates on PC3, and Eucarida sequences separated sampling dates along PC4 (Fig. 4).

The log-transformed COI and V9 sequence numbers from the same stomach samples were positively correlated for the 3 most important prey groups, Eucarida (coefficient of multiple determination [R^2]=0.35, $P<0.001$), Polychaeta ($R^2=0.57$, $P<0.001$) and Amphipoda ($R^2=0.48$, $P<0.001$), but not for the other, less abundant prey groups (Fig. 5).

Discussion

Through DNA metabarcoding of stomach contents, we identified the diets of juvenile black sea bass recently settled in LIS, corroborating and refining the general view of this species as a benthic omnivore that consumes mostly crustaceans, including shrimp, crab, and amphipod species (Richards, 1963; Link, 1980; Sedberry, 1988). The study site is geographically close to Narragansett Bay, in Rhode Island, where Taylor et al. (2023) found mainly amphipod, shrimp, crab, nematode, and fish species in the diets of juvenile black sea bass by using morphological inspection.

Comparison of both studies revealed several noteworthy contrasts.

First, Taylor et al. (2023) found the diet of small juvenile black sea bass (<50 mm TL) to be dominated by amphipods (83%) over shrimp species (7%), whereas the results from our study indicate the opposite, a consistent dominance of shrimp species over amphipods and polychaetes. Because both crustacean groups have hard, chitinous carapaces, they likely remain detectable in fish stomachs (Buckland et al., 2017), indicating that juvenile black sea bass in Mumford Cove consumed more shrimp than similarly sized black sea bass in Narragansett Bay. The sevenspine bay shrimp and zostera shrimp are both very common species in coastal waters of the Northwest Atlantic Ocean (Price, 1962; Haefner, 1979; Modlin, 1980). These 2 shrimp species have been found to be particularly abundant in habitats, such as Mumford Cove, with dense subaquatic vegetation like the eelgrass *Zostera marina* (Bologna, 2007; Vaudrey et al., 2010; H. Baumann, personal observ.), a type of habitat not included in the varied habitats and substrates sampled by Taylor et al. (2023). Juvenile black sea bass may prefer shrimp, when abundant, as an energetically denser prey over smaller amphipods. Future work could examine this further by testing whether diets of juveniles of other common fish species in this region, such as the cunner (*Tautoglabrus adspersus*) and tautog (*Tautoga onitis*), have the same spatial variation in prey as the diets of juvenile black sea bass (Taylor et al., 2023).

The occurrence of polychaetes in stomachs of black sea bass is a second notable contrast between the studies, given that Taylor et al. (2023) reported generally low incidence of this class (<2%) but we found substantially greater percentages of polychaete sequences in samples from stomachs of similarly sized juvenile black sea bass (10% on average). The 2 identified polychaete species, sand worm and clam worm, are common benthic organisms in habitats of the

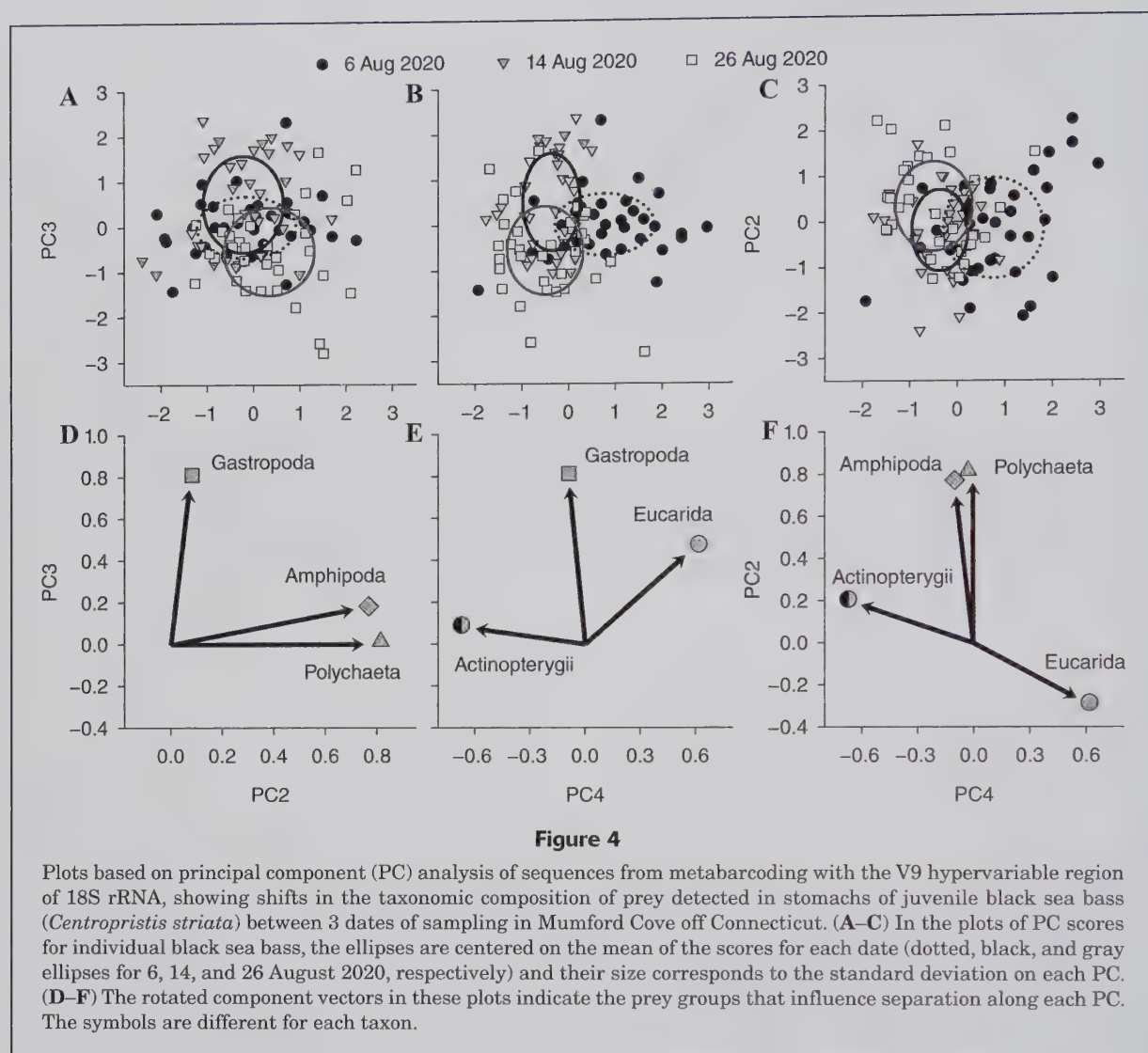


Figure 4

Plots based on principal component (PC) analysis of sequences from metabarcoding with the V9 hypervariable region of 18S rRNA, showing shifts in the taxonomic composition of prey detected in stomachs of juvenile black sea bass (*Centropristis striata*) between 3 dates of sampling in Mumford Cove off Connecticut. (A–C) In the plots of PC scores for individual black sea bass, the ellipses are centered on the mean of the scores for each date (dotted, black, and gray ellipses for 6, 14, and 26 August 2020, respectively) and their size corresponds to the standard deviation on each PC. (D–F) The rotated component vectors in these plots indicate the prey groups that influence separation along each PC. The symbols are different for each taxon.

Northwest Atlantic Ocean (Whitlatch, 1982; Lopez et al., 2014), but genuine differences in polychaete abundances could exist between the study sites in Mumford Cove and Narragansett Bay. However, we suggest that polychaetes compose a more substantial fraction of the prey of juvenile black sea bass than previously known. Polychaetes are soft-bodied prey that may disintegrate rapidly in fish stomachs, making them difficult to detect through traditional morphological inspection (Buckland et al., 2017). In contrast, metabarcoding can be used to classify disintegrated tissue; therefore, we argue that this approach is better at detecting polychaetes. Similarly, this difference in detection between methods may also apply to other soft-bodied prey groups that are orders of magnitude rarer. For example, Hemichordata were detected in low sequence numbers through metabarcoding of the V9 region in our study but were absent or undetected in stomachs of juvenile black sea bass captured from Narragansett Bay.

The third important contrast between studies concerns the occurrence of amphipod prey in stomachs of juvenile

black sea bass. Taylor et al. (2023) found amphipods, including species from both Gammaridae and Caprellidae, to be the dominant prey (83%) of small juvenile black sea bass, a result that is consistent with analyses by Sedberry (1988) of the stomach contents of small black sea bass (<100 mm in standard length) caught in habitats off South Carolina, Georgia, and northeastern Florida in the North Atlantic Ocean. In contrast, in our study, much smaller average proportions of amphipod sequences were found in samples from stomachs of black sea bass, and these sequences belonged almost exclusively (97%) to a single species of Aoridae, *G. japonica*. This finding is noteworthy because *G. japonica* is native to the western Pacific Ocean (off northern Japan; Stephensen, 1938). Over the past 60 years, it has invaded and established itself first in waters of the Northeast Pacific Ocean off California, then in waters of the Northeast Atlantic Ocean and in regions off Australia, and most recently in the Northwest Atlantic Ocean.

Specifically for Long Island Sound, the first evidence of *G. japonica* was reported in 2013 (Trott et al., 2020), and

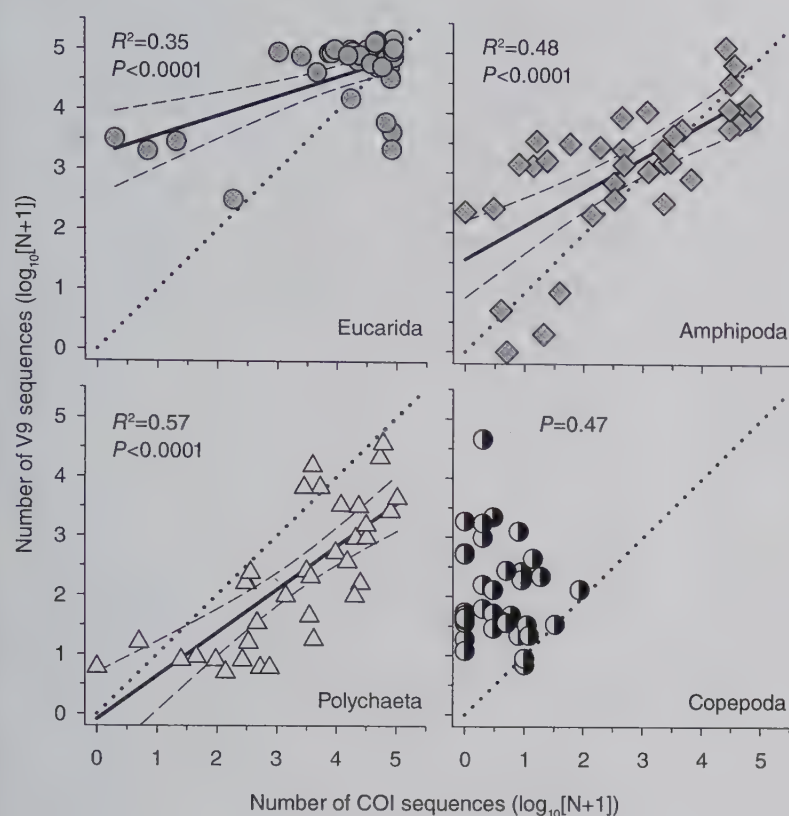


Figure 5

Relationship between log-transformed numbers of sequences ($\log_{10}[N+1]$) from metabarcoding with the mitochondrial cytochrome c oxidase subunit I (COI) gene region and those from metabarcoding with the V9 hypervariable region of 18S rRNA for the 4 major prey groups, Eucarida, Amphipoda, Polychaeta, and Copepoda, detected in stomach samples from 35 juvenile black sea bass (*Centropristis striata*) collected in Mumford Cove off Connecticut in August 2020. Symbols are data points for individual juveniles, and the solid and dashed lines depict linear regression lines and their 95% confidence intervals. The dotted line in each graph represents the 1:1 line. The coefficients of multiple determination (R^2) and P -values from regression analysis are provided.

yet, at our study site, this invasive amphipod now appears to have become the exclusive prey for small juvenile black sea bass (<70 mm TL). This result is especially significant, given that other amphipods, especially caprellids, remain highly abundant in Mumford Cove (H. Baumann, personal observ.). The reference database used for the metabarcoding analysis confirmed our findings with geolocation and reference sequences for 2 species in the North Atlantic Ocean (out of 10 species of *Grandidierella* worldwide). Both species, *G. bonnieroides* (Gulf of Mexico and Florida) and *G. japonica* (North Atlantic Ocean), have geolocation records. However, more efforts are needed to generate COI reference sequences (or barcodes) of other amphipod prey species.

Taylor et al. (2023) reported a notable proportion (13% occurrence) of nematodes among prey items in the guts of black sea bass and commented that these could include endoparasites or pseudoparasites, which have been

documented to live in fish intestines or stomachs (Buchmann, 2012; Abolafia et al., 2015). In contrast, we found negligible numbers of nematode sequences (43 sequences across 8 samples) in samples from stomachs of black sea bass, perhaps, because we focused on the smallest, most recently settled individuals that may not have had sufficient exposure to prey containing nematodes. Similarly, because of our focus on the smallest juveniles, stomach samples were analyzed for very few juvenile black sea bass ≥ 100 mm TL. Nonetheless, the stomachs of these large individuals contained significantly fewer amphipods and polychaetes (on the basis of metabarcoding of the V9 region) and more fish prey (on the basis of metabarcoding of the COI region) than the stomachs of the smaller fish (20–70 mm TL) in our study, a result that is consistent with Taylor et al. (2023) reporting a steep decline in the importance of amphipods in juvenile black sea bass ≥ 50 mm TL and a greater occurrence of fish and decapods in their stomachs. Both fish species, the northern pipefish and Atlantic silverside, detected as prey in our study are among the most common species in Mumford Cove during summer (e.g., Pringle and Baumann, 2019).

In aquaculture or laboratory settings, cannibalism is known to occur among juvenile black sea bass (Watanabe et al., 2021; H. Baumann and M. Zavell, personal observ.), but cannibalism has not been reported from previous studies in which stomach contents of black sea bass collected in the field were analyzed (Sedberry, 1988; Taylor et al., 2023). Cannibalism cannot be confirmed through

molecular approaches, given the likelihood that stomach homogenates used to extract DNA will also contain traces of the predator's stomach tissue. This limitation is particular to the use of the V9 gene marker, which cannot be used to identify prey items more specifically than the class Actinopterygii and therefore results in the grouping of sequences of potential teleost prey with sequences of the predator. Another limitation is the potential for detecting DNA sequences of taxa consumed by the prey of the organism of interest; it is not possible through metabarcoding to distinguish between sequences for taxa in the guts of the prey and sequences for taxa in the stomach of the predator. In our study, this limitation may be apparent in the large number of detected Copepoda sequences, given that settlement of juvenile black sea bass entails transition from pelagic, planktivorous larvae to benthivorous adults that are unlikely to consume copepods. This issue could be effectively resolved with future laboratory experiments in

which natural plankton would be offered to newly settled juvenile black sea bass.

Although we found that the use of both COI and V9 gene markers produced qualitatively consistent results for the 3 most abundant prey groups (Eucarida, Amphipoda, and Polychaeta), the choice of gene regions for metabarcoding analysis can potentially affect the patterns of biodiversity observed (Djurhuus et al., 2018; Questel et al., 2021). The 313-bp region of the mitochondrial COI gene targeted in our study (Geller et al., 2013; Leray et al., 2013) has been widely used for metabarcoding analysis of marine metazoan diversity (Hirai et al., 2015; Blanco-Bercial, 2020; Govindarajan et al., 2021). Through metabarcoding of the COI gene region, we identified species of some—but not all—groups of organisms and may not have detected some prey that are also overlooked in morphological analyses of gut contents. In contrast, the V9 region can be used to reliably classify sequences at varying taxonomic levels for a broad range of phyla (de Vargas et al., 2015), but it lacks sufficient sequence variation for species identification. In this study, use of the V9 region in metabarcoding resulted in detection of 3 groups (Ascidiacea, Ostracoda, and Hemichordata) that were not detected by using the COI region, indicating the advantage of using multiple gene regions for metabarcoding analysis to fully capture the diversity of prey of juvenile black sea bass.

Accurate classification of prey groups or species through metabarcoding also relies on the geographic coverage and completeness of reference sequence databases. These databases must be accurately curated and contain sequences from specimens identified morphologically by taxonomic experts (Bucklin et al., 2021). In our study, we used a tailored database created, following the method described by Blanco-Bercial (2020), from the data set of small subunit sequences in release 132 of the SILVA database that includes eukaryotic marine sequences from GenBank (available from website) for the V9 region. Similarly, the MetaZooGene Atlas and Database is continuously updated, providing confidence in the use of COI sequences from that database in classification of prey taxa based on results from metabarcoding (Bucklin et al., 2021; O'Brien et al., 2024).

Conclusions

Given the rapid environmental and ecological changes unfolding in LIS and other coastal regions of the Northwest Atlantic Ocean (Howell and Auster, 2012; Crosby et al., 2018; Snyder et al., 2019), monitoring, sampling, and diet studies of species, such as the black sea bass, are critical tools for prediction of changes to local and regional food webs. The combination of rapid warming and altered ecological interactions (i.e., predation and competition) may result in further displacement of cold-water species that may be outcompeted by warm-water species like the black sea bass (Mercaldo-Allen et al., 2020). In this study, we identified prey groups and species that could be affected by large, mid-summer settlement pulses of black

sea bass in LIS habitats, with consequences for other fish species with similar diets, including juvenile cunner and tautog (Taylor et al., 2023). Future studies of the effects of black sea bass on food webs in LIS will require efforts to sample black sea bass of a larger size spectrum, from a range of habitats and seasons and with concurrent sampling of associated benthic communities. The results of this study indicate that molecular approaches, such as DNA metabarcoding of stomach contents, can play an important role alongside morphological approaches in investigating fish diets and their effects on marine ecosystems.

Resumen

La abundancia de cabrilla negra (*Centropristis striata*) ha aumentado en las aguas costeras del noreste de Estados Unidos, y las implicaciones de ese cambio para las redes tróficas regionales siguen siendo poco conocidas. Utilizamos la metabarcodificación del ADN de 2 regiones génicas, la región mitocondrial citocromo c oxidasa subunidad I (COI) y la región hipervariable V9 del ARNr 18S, para clasificar las presas en el contenido intestinal de juveniles de cabrilla negra colectados en un hábitat cercano a la costa en Long Island Sound (LIS). Utilizando los números de secuencia de las regiones COI y V9, descubrimos que el contenido intestinal estaba dominado por crustáceos del superorden Eucarida, seguido de 2 especies de poliquetos y un anfípodo invasor, *Grandidierella japonica*. Los bivalvos, gasterópodos y ostrácodos fueron menos abundantes. En los estómagos de 2 cabrillas negras grandes se observaron (>10% de las secuencias) restos del pez pipa del norte (*Syngnathus fuscus*) y el pejerrey del Atlántico (*Menidia menidia*). Se observó una gran variabilidad en el número de secuencias entre individuos, tanto para la región COI como para la región del gen V9. La composición taxonómica de las presas también difirió: los eucaridos contribuyeron con >87% de las secuencias del gen V9 en muestras de 51 estómagos, los anfípodos con >50% de las secuencias en muestras de vísceras de 21 peces, y los poliquetos dominaron con >75% de secuencias en muestras de 6 estómagos. Concluimos que, durante los pulsos de asentamiento, los juveniles de cabrilla negra pueden competir con otros depredadores bentónicos por las presas de decápodos, poliquetos y anfípodos en los hábitats cercanos a la costa de LIS.

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Abstract—Gonad histology complements research on the life history of fish species and provides greater accuracy and precision than macroscopic characterization of the gonad for determining patterns of oogenesis at the cellular level and maturation at the individual level. In a fishery context, histology improves estimation of mature and spawning stock biomass, identification of spawning seasonality and grounds, and preselection of specimens for calculation of annual fecundity. However, in most studies, only a single staining method (hematoxylin and eosin) has been used. In this study on 3 taxonomically diverse species, using 4 different staining methods of varying complexity (degree of counterstaining), we compared confidence levels in identification of 8 stages of oogenesis, the presence and level of degradation for postovulatory follicles, and atretic (vitellogenic) germ cells. As anticipated, the method involving the least expensive, monochromatic stain provided the lowest level of confidence, whereas the most expensive and complex counterstaining method provided the highest confidence level, with hematoxylin and eosin staining and another simple counterstaining method in between them. The effect of staining method was most evident for identifying cortical alveoli, which can affect estimation of size or age at maturity, and for identifying postovulatory follicles, which can affect estimation of spawning frequency. These results are broadly applicable for determining best practices.

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A comparison of 4 histological staining methods for revealing oocyte development, atresia, and postovulatory follicles in 3 fish species

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The economic value of fish has long attracted interest in understanding how fish reproduce in the wild and through aquaculture. By the second half of the 19th century, a fundamental understanding of fish reproductive dynamics was emerging, specifically knowledge of how reproductive tissue in gonads generates cohorts of germ cells provisioned with yolk (Cunningham and Oxon, 1897; Fulton, 1898; Wallace, 1903). A powerful tool for this work was histology, in which gonad tissue was fixed, thinly sectioned, and stained for examination under a microscope. Early stains were adapted from the textile dyeing industry or from simple chemicals available in the laboratory (e.g., silver nitrate) (Titford, 2009; Riva et al., 2014). The use of even such basic stains yielded insight into major stages of oogenesis, such as the transition from mitotic oogonia to meiotic oocytes, the transition from unyolked to yolked oocytes, and the transition from oocytes to mature eggs. Recognition of how histochemical stains worked grew

exponentially during the 20th century (Wick, 2012; Riva et al., 2014). Although many stains developed more than a century ago are still in use today, others have been replaced or discontinued because of their toxicity (Titford, 2009). Eventually, some stains became widely used because they were easy to formulate, were inexpensive, or provided improved resolution (e.g., hematoxylin and eosin [H&E]) (Javaeed et al., 2021), whereas others were more complex to execute but provided even finer resolution for examining subcellular features (e.g., trichrome stains) (Titford, 2009; Alturkistani et al., 2016).

Today, many different histological stains are available to highlight specific structures and features in cells and tissues of vertebrates, including fish species (Morrison, 1990; Grier et al., 2018; Smith et al., 2018; Uribe et al., 2019). Although different staining methods may yield different levels of identification and understanding, histological analysis of gonads with even

basic stains is considered superior to macroscopic methods in determining reproductive status (West, 1990; Ferreri et al., 2009; McBride et al., 2013). For these reasons, many fish biologists use gonad histology to increase the precision and accuracy of maturity estimates (McBride et al., 2013), spawning seasonality (Press et al., 2014; McElroy et al., 2022), and reproductive strategies for life history studies and stock assessments (Lowerre-Barbieri et al., 2023).

Gonad histology is, however, costly. In fishery science, we often characterize reproductive traits at the population level, in contrast to how, in medical science, doctors diagnose conditions of individuals. Consequently, even when subsampling, fishery biologists may examine the gonads of hundreds to thousands of individuals per species. They use gonad histology to examine specific features, such as the appearance of cytoplasmic inclusions or the migration of the nucleus, with the intent to estimate parameters, such as spawning seasonality, size at maturity, or the number of eggs produced per unit of time. Because of the large sample sizes required for fisheries population studies, it is necessary to optimize the cost-benefit ratio of different staining methods when using gonad histology. In other words, it is necessary to choose the most reliable method that can be used for the lowest cost. Such choices may be quite different between working in a research mode on species with yet unresolved patterns of oocyte development and reproduction and more routine monitoring of a familiar species. In a research mode, for example, for little additional cost, a complex stain can reduce the ambiguity of cytoplasmic features in the germ cells of an unfamiliar species or reduce the risk of biased or imprecise estimates of reproductive potential. Alternatively, in an operational mode for routine work, a simple stain suitable for a familiar species can allow more efficient processing of high numbers of slides, reducing the cost per slide.

Despite the diversity and utility of the different stains available, we determined that H&E is by far the most commonly used stain for most fish (and animal) studies (Riva et al., 2014), on the basis of a search in 2023 and 2024 of published papers on studies of fish reproduction in which gonad histology was used (number of articles=66) (Fig. 1). Only a few other stains, notably Mallory's trichrome, Masson's trichrome, periodic acid Schiff's (PAS) with counterstains of iron-hematoxylin and metanil yellow, and toluidine blue (TB) in sodium tetraborate, had been applied to fish species. The benefits of using H&E include a detailed view of intracellular and extracellular components and morphology of tissues, providing diagnostic information even when additional stains are applied for specific features. In clinical settings, H&E staining is used initially as the standard or routine method and additional stains (often referred to as *special stains*) are used only when further information is needed. The routine nature of H&E staining makes it amenable to use in fully automated systems to efficiently process large quantities. Although many complex stains provide increased contrast, clarity, and certainty in identifying key features, we postulate that they are too costly for routine monitoring programs. The cost of special stains can vary widely and can

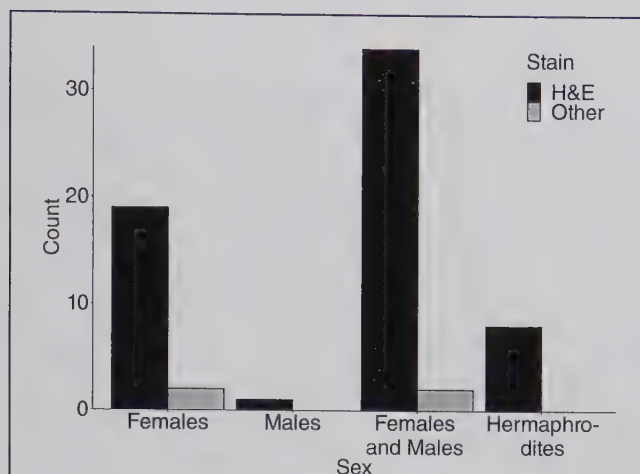


Figure 1

Number of research papers published in 2023 and 2024 on studies of teleost reproduction that involved the use of histological stains on gonad tissue, by sex of fish and category of stain. The 66 publications found in our literature search are classified on the basis of the sex of fish examined: females, males, both female and male individuals of gonochoristic species, or hermaphrodites. The papers are also separated into 2 categories based on the stains used in the studies: hematoxylin and eosin (H&E) or *Other*, which includes Mallory's trichrome, Masson's trichrome, a combination of periodic acid Schiff's, iron-hematoxylin, and metanil yellow, and a combination of toluidine blue and sodium tetraborate.

be equal or similar to the cost of H&E—or more than double the cost.

The objectives of this study were to evaluate the costs and benefits of 4 staining methods of varying complexity (levels of counterstaining) (Table 1) and to provide information that can help others determine the optimal stain considering the objective and resources available for their studies. The methods included one with a monochromatic stain and 3 techniques that involved counterstaining. Variation within each sample was minimized by applying all 4 staining methods to thin-tissue sections of the same sample (i.e., the same histology block). We chose 3 fish species that are economically important and phylogenetically diverse in the Northwest Atlantic Ocean: Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), and yellowtail flounder (*Myxopsetta ferruginea*). All 3 species have been previously reported to have group-synchronous oocyte development, with the larger, vitellogenic cohort of oocytes easily distinguishable leading up to spawning (Murua and Saborido-Rey, 2003). The spawning pattern for Atlantic herring differs from those of haddock and yellowtail flounder. Atlantic herring are total spawners, meaning they ripen, ovulate, and shed the whole annual clutch of eggs at once or over a short period of time (hours to days) (van Damme et al., 2009; Kennedy et al., 2011a; McPherson et al., 2011; Bucholtz et al., 2013). Haddock and yellowtail flounder are batch spawners,

Table 1

Type of oocyte development, spawning pattern, length ranges, stocks, collection times, sample sources, and gonadosomatic index (GSI) values for the female Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), and yellowtail flounder (*Myxopsetta ferruginea*) for which gonad tissue was analyzed by using 4 histological staining methods. Also provided are the numbers of fish from which tissue samples were taken, by species and stain. The 4 stains are toluidine blue, hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Price ranges for each stain, given in parentheses, are for the costs per slide of processing, embedding, and staining tissue samples at histology laboratories that we have used recently (in 2022–2024). Samples were taken from stocks in the Gulf of Maine (GOM), Georges Bank (GB), and Southern New England (SNE). Sample sources were bottom-trawl surveys (BTS) of the NOAA Northeast Fisheries Science Center (NEFSC), the Study Fleet program of the NEFSC Cooperative Research Branch (CRB) and fishery-dependent sampling of the Maine Department of Marine Resources (MEDMR). Additional information on the individual fish analyzed is provided in the Supplementary Table (online only). FL=fork length; TL=total length.

Characteristic	Species		
	Atlantic herring	Haddock	Yellowtail flounder
Oocyte development	Group-synchronous	Group-synchronous	Group-synchronous
Spawning pattern	Total	Batch	Batch
Length	170–263 mm FL	408–676 mm FL	306–440 mm TL
Stock	GOM, GB	GOM, GB	GOM, GB, SNE
Years sampled	2015, 2016	2013, 2015	2010–2012, 2018, 2019
Months sampled	Sept.–Nov.	Jan.–March	March–June, Nov.
Sample sources	NEFSC BTS and CRB, MEDMR	NEFSC CRB	NEFSC CRB
GSI	<0.01–8.5	0.6–11.8	3.3–20.6
Stain (price per slide)	Number of fish used in tissue analysis		
Toluidine blue (\$16.25–\$17.25)	11	10	9
H&E (\$11.00–\$12.25)	11	10	9
PAS (\$13.80–\$17.25)	11	10	9
PAS-MT (\$19.00–\$23.00)	11	10	9

ripening, ovulating, and shedding multiple batches of eggs over a longer period of time (weeks to months) (Robb, 1982; Howell, 1983; Clay, 1989; Tronbøl et al., 2022).

We selected mature individuals of each species that best represented certain critical phases of oogenesis. Mature fish have the potential to have oocytes in multiple stages of development, including oogonia in nests, oocytes in various stages up to ovulation, and eggs, in addition to postovulatory follicles (POFs) and atresia, potentially present at one time (Table 2), in contrast to immature fish that have only oocytes in the primary growth stage. We included individuals in an early stage of maturing, with oocytes in the cortical alveolar stage; such oocytes can be difficult to detect with some histological stains. For example, Blazer (2002) notes that “[cortical] alveoli are produced within the oocyte and stain positively with a PAS (periodic-acid Schiff) stain,” implying that these features may not show up without the use of a PAS stain. This reliance on the application of a PAS stain is important because some researchers do not consider cortical alveoli, which are produced endogenously within the oocyte (at the

germ cell level), to be indicative of a mature female (at the individual level) whereas others do (see the “Discussion” section in Brown-Peterson et al., 2011; Prince et al., 2022). Another major step, vitellogenesis, according to Blazer (2002), “involves the sequestering (from the blood) and packaging of hepatically-derived [sic] vitellogenin,” a step considered by some to be a threshold for maturity. The presence of oocytes in later additional stages, such as nucleus migration and ovulation, indicates that the fish is entering the second arrest phase (meiosis II), generally a sign that spawning is imminent for that individual. The presence of POFs indicates that the fish had already released at least one batch of eggs, if a batch spawner, or at least started to release its singular batch, if a total spawner. The presence and type of atretic germ cells was also recorded because these cells are often associated with past spawning activity, which may be particularly relevant when trying to distinguish an immature female from a mature female outside of the spawning season.

Because individual variation was minimized and fish in a range of maturity stages for 3 phylogenetically diverse

Table 2

Description of microstructural features recorded during readings of slides of stained gonad tissue from Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), and yellowtail flounder (*Myxopsetta ferruginea*) caught in the Northwest Atlantic Ocean during 2010–2019 (for details of collections, see Table 1). These features were used to identify the following 8 stages of oocyte development: perinucleolar, cortical alveolar, early vitellogenesis, late vitellogenesis, germinal vesicle migration, germinal vesicle breakdown, hydration, and ovulation. The stages can be categorized into 4 phases, primary growth, secondary growth, early maturation, and late maturation. Postovulatory follicles (POFs), follicular atresia, blood vessels, muscle bundles, and tunica are also described.

Microstructural features	Histological criteria
Primary growth	
Perinucleolar	Perinuclear oocytes have multiple nucleoli oriented around the periphery of the germinal vesicle. Chromatin nucleoli are observed at the same time and classified with perinucleolar oocytes as primary growth, as per Blazer (2002). Chromatin-nucleolus oocytes have a single or a few prominent nucleoli in the germinal vesicle and are highly basophilic.
Secondary growth	
Cortical alveolar	Cortical alveoli first appear in the cytoplasm along the periphery of the oocyte as small, circular, white inclusions (Blazer, 2002). Cortical alveoli will then fill with dark dots and increase in number and, as vitellogenesis proceeds, the cortical alveoli are “pushed” to the periphery of the oocyte (Blazer, 2002).
Early vitellogenesis	Lipoprotein yolk globules begin to appear around the periphery of the germ cell and advance inward toward the germinal vesicle. Cortical alveoli are still present around the zona pellucida.
Late vitellogenesis	Lipoprotein yolk globules continue to fill the whole cytoplasm and grow in size. A small ring of cortical alveoli can still be seen near the zona pellucida.
Early oocyte maturation	
Germinal vesicle migration	After the lipoprotein yolk globules fill the cytoplasm, the germinal vesicle migrates toward the animal pole of the zona pellucida.
Late oocyte maturation	
Germinal vesicle breakdown	While the germ cell remains in the follicle, the germinal vesicle breaks down and the lipoprotein yolk globules fuse, eventually becoming a hyaline, homogeneous mass (Blazer, 2002).
Hydration	Remaining within the follicle, yolk globules become fused and water content is absorbed, increasing the size of the oocyte significantly.
Ovulation	The hydrated oocyte is released into the lumen from the follicle cells and overlying germinal epithelium, leaving behind the POF and becoming an egg.
Postovulatory follicle	
Recent	A complex structure consisting of granulosa cells (inner layer) and theca cells (outer layer). The columnar granulosa cells are typically separated from the theca. The follicle is loosely arranged and irregular in shape with a lumen larger than a perinucleolar oocyte.
Intermediate	The granulosa and theca cell layers remain distinguishable. The follicle is more compact and approximately the size of a perinucleolar oocyte. The lumen is still visible but much smaller in size than recent POFs.
Old	The 2-layer structure may be identifiable in some instances, but cell integrity is greatly deteriorated. The follicle is almost entirely collapsed without a distinguishable lumen and is smaller in size than a perinucleolar oocyte.
Follicular atresia	
Alpha	The germinal vesicle disintegrates, the zona pellucida breaks down, the basement membrane remains intact, and lipoprotein yolk globules are present in early and late vitellogenic oocytes.
Beta	Internal oocyte components (lipoprotein yolk globules and cortical alveoli) are digested through phagocytosis. Oocyte has a bubble-like appearance.
Other structures	
Blood vessels	Blood vessels or capillaries identified within the muscle tissue.
Muscle bundles and connective tissue	The presence of muscle bundle or connective tissue.
Tunica and tunica thickness	The presence of tunica and tunica thickness. The thickness of tunica varies between species and degree of ovarian development, and a measurement is recorded if present.

species were selected, the results of this study should be generally applicable to a wide range of species and, therefore, of interest to others considering the advantages and disadvantages of different staining methods for gonad histology.

Materials and methods

For this comparison study, we focused on ovarian (female) histology. As noted in the “Introduction” section, we selected 3 fish species of the Northwest Atlantic Ocean—Atlantic herring, haddock, and yellowtail flounder—to generalize phylogenetic diversity. We selected 8 stages of oogenesis, identifying major transitions of oocyte development (Table 2), and evaluated different methods of stain preparation by using samples of the same individuals from each species to minimize individual variation.

Individuals were selected from recent (Press et al., 2014; McElroy et al., 2016; Wuenschel and Deroba, 2019) and ongoing projects to best represent the variety of oocyte development stages in mature females within each species. These individuals were collected from fishery-independent (NOAA Northeast Fisheries Science Center [NEFSC] seasonal bottom-trawl surveys) and fishery-dependent (NEFSC Cooperative Research Branch Study Fleet) sources. The total length or fork length and the weight of fish were measured to the nearest millimeter and 0.1 g. The weight of gonads was measured to the nearest 0.001 g. The gonadosomatic index (GSI) for fish was calculated as follows:

$$GSI = 100(\text{gonad weight} / [\text{total weight} - \text{gonad weight}]).$$

Mature fish were chosen because they had oocytes in multiple stages of development present at one time (Table 2). The progression through the cortical alveolar and vitellogenic stages can be protracted in cold-water species, in extreme cases taking several years (Kennedy et al., 2011b; Press et al., 2014; McBride et al., 2022a, 2022b); therefore, these stages do not represent functional maturity if observed a year or more before a fish spawns for the first time (Lefebvre and Field, 2015; Pacicco et al., 2023). Once oocytes complete development, release from the follicle to become eggs, and are spawned, the presence of POFs helps in determining if a fish had successfully spawned (total) or had started to release some eggs (batch, partially spent).

Gonad samples were preserved in 10% buffered formalin for more than 1 month before the cutting of cross sections of 3–5 mm, and the cross sections were transferred into cassettes and sent to a histology lab. In the histology lab, tissue was dehydrated in an increasing series of ethyl alcohol concentrations, embedded in paraffin, and sectioned to 5 µm (Shaw et al., 2012). The 4 histological stains ranged from a simple monochromatic stain, TB, to the most complex stain, a combination of PAS and Mallory's trichrome (PAS-MT).

Toluidine blue is a basic thiazine metachromatic dye and binds to nuclear material of tissues with DNA and RNA content (Sridharan and Shankar, 2012). Slides stained

with TB appear monochromatic in an array of blue hues with a nucleus of a violet-like color. Although TB is theoretically a simpler stain than H&E, the cost for TB-stained slides from production laboratories may be comparable to the cost for slides stained with H&E, unless a laboratory has an automated process for this stain. The standard TB protocol is very simple, with a single short dip in the stain solution followed by a rinse under running water; therefore, a less expensive option for some researchers may be to obtain sectioned, unstained slides that they can stain themselves. The method of staining with TB is suitable for circumstances in which laboratory resources are limited and is the standard technique applied in studies of coral-reef fishes carried out at remote field locations (Longenecker and Langston, 2018; Longenecker et al.¹).

Hematoxylin and eosin was selected because of the familiarity that most histologists and fisheries scientists have with this stain as the standard or routine stain used in fisheries research (Shaw et al., 2012). In staining with H&E, slides were initially stained with the hematoxylin component, which has a deep blue-purple color that binds to nucleic acids. Next, the counterstain eosin was applied, staining the cytoplasm and extracellular matrix in varying degrees of pink. With the H&E staining method, tunica and muscle tissue appear light pink, the nucleus is pink, and the zona pellucida and lipoprotein yolk globules become a dark pink. Postovulatory follicles in all stages appear in a variety of pink hues.

A more complex stain, PAS can be used to discern the presence of carbohydrates and carbohydrate compounds. In the initial reaction, the periodic acid acts as an oxidizing agent, producing Schiff reactive aldehyde groups. In the second step, the tissue sections react with the Schiff's reagent to produce a bright magenta color. Hematoxylin is then used as a counterstain to reveal other tissue elements. With PAS staining, tunica and muscle tissue appear purple and oocytes have a light-purple nucleus, dark-purple cytoplasm, and purple lipoprotein yolk. The POF stages are stained from light to dark purple, with intermediate and old POFs having a definite magenta color.

The most complex stain we evaluated was PAS-MT. For this stain, the sample initially goes through the PAS staining protocol (as described in the previous paragraph), before application of the trichrome stain that consists of aniline blue, acid fuchsin, and orange G to help reveal more detail (Mallory, 1900; Titford, 2009). Although this stain is not widely used in fisheries research, per our literature searches (see Fig. 1), we have published comprehensive plates depicting oogenesis with this stain for several species: winter flounder (*Pseudopleuronectes americanus*) (Press et al., 2014), American shad (*Alosa sapidissima*) (McBride et al., 2016), Atlantic herring (Wuenschel and

¹ Longenecker, K., R. Langston, and E. C. Franklin. 2020. Standard operating procedure for histology-based rapid reproductive analysis of tropical fishes, 93 p. Report prepared by the University of Hawaii at Manoa for Tetra Tech's Supplemental Technical Assistance to the USAID SEA Project and Walton Family Foundation. Univ. Hawaii Manoa, Honolulu, Hawaii.

Deroba, 2019), Atlantic halibut (*Hippoglossus hippoglossus*) (McBride et al., 2022a), Atlantic wolffish (*Anarhichas lupus*) (McBride et al., 2022b), and cusk (*Brosme brosme*) (McElroy et al., 2022). We obtained the formula for this stain (Witthames²), which has been used to distinguish old POFs that have a basal membrane from atretic cells in Atlantic cod (*Gadus morhua*) (Witthames et al., 2010), and provide it in the Supplementary Materials (online only) because it is not widely published in histology handbooks. The PAS-MT stain reveals much more contrasting detail than the other stains used in this study, highlighting critical characteristics of oogenesis that help to determine the maturity and spawning phases of individual fish. With PAS-MT staining, the cytoplasm is light gray, the nucleus has a blue hue, and the lipoprotein yolk globules are red. The POFs are also very distinct, appearing dark purple, and can be easily identified and categorized into different stages.

Histology slides were scanned with an Ocus 40³ digital microscope (model MGU-00003; Grundium, Tampere, Finland) and read by using the software Aperio ImageScope, vers. 12.4.6.5003 (Leica Biosystems, Nussloch, Germany). Three readers analyzed each histology slide independently. Expertise levels differed slightly among readers, but each of them had >10 years of experience reading histology of multiple species, primarily using PAS-MT, PAS, and some H&E preparations, but with exposure and familiarity to all stains through workshops and published literature. Readers recorded the most advanced stage of oocyte development and all other oocyte stages present, the presence of POFs and the stage of each POF (recent, intermediate, or old), the presence of atresia and the stage of atresia (alpha and beta), the presence of blood vessels and muscle bundles, and the presence of tunica and tunica thickness (Table 2). Each reader annotated representative stages of oocyte development and other features on the slides. In addition, the confidence level (high, medium, or low) was indicated for each recorded feature. High confidence was recorded if the reader had 100% certainty in identification of the microstructural feature, medium confidence was recorded if the reader was 50% certain in identification of the feature, and low confidence was recorded if the reader was unsure but tentatively noted the feature as present. In addition, to get a more general sense of the readability of each slide, each reader also recorded the overall confidence in the interpretation of each slide as high, medium, or low.

Results

For each of the 30 gonad samples, we evaluated 4 separate slides, each prepared with 1 of the 4 histological stains (Suppl. Table) (online only). A total of 3265 features

were recorded from the 360 slide readings (3 readers×120 slides). Herein, results are summarized first by species, then confidence levels for identification of various features (i.e., oocyte development stages, POFs, and atresia) are described, and finally a comparison of stains and features across species is provided.

Oogenesis

Atlantic herring Across all 4 staining methods, oocytes in 7 of the 8 stages of oogenesis (all but ovulation) were identified in the slides for selected samples of Atlantic herring, with the presence of oocytes in stages from primary growth through hydration and other microstructural features, such as degradation of POFs and follicular atresia, recorded. Primary growth oocytes, which included those in chromatin nucleolar and perinucleolar stages, were present in every slide. Secondary growth stages, beginning with the presence of cortical alveoli, were detected with all stains (Fig. 2, A–D). Cortical alveoli were present initially around the periphery of the oocyte as clear circular structures, with darker staining inclusions evident in all slides but the ones made with the TB stain. The cortical alveoli increased in number and size, eventually being pushed to the outer edge of the cytoplasm. Early stage vitellogenesis (Fig. 2, E–H) began when lipoprotein yolk globules began to form around the periphery of the oocyte, eventually increasing in size and number and filling the cytoplasm with yolk. Once filled with lipoprotein yolk globules, the germinal vesicles migrated toward the zona pellucida of the oocyte, marking final oocyte maturation (Fig. 2, I–L). Hydration was the most advanced stage of oogenesis we recorded in Atlantic herring, identified when the lipoprotein globules were completely fused and when water uptake increased the size of the oocytes but they remained within the follicle.

Three stages of POFs were identified in Atlantic herring across all 4 methods of staining (Fig. 3). Recent POFs were irregular in shape and, in slides with some stains (PAS and PAS-MT), the theca and granulosa layers could be more easily distinguished (Fig. 3, G and H). Recent POFs were larger than a perinucleolar oocyte and had a large lumen (Fig. 3, A–D). The 2 layers were still visible in intermediate POFs (Fig. 3, E–H), which had a collapsed lumen and were smaller. Old POFs were significantly smaller than a perinucleolar oocyte with a completely collapsed lumen, and the theca and granulosa layers were almost completely indistinguishable (Fig. 3, I–L).

Follicular atresia was categorized as alpha or beta atresia for Atlantic herring (Fig. 4). Alpha atresia was visible by the breakdown of the zona pellucida and the disintegration of the germinal vesicle (Fig. 4, A–D). Advanced (or late) alpha atresia was identified with the PAS-MT stain (Fig. 4H) because of the visible presence of lipoprotein yolk globules in a smaller, partially resorbed oocyte. Beta atresia, defined by the absence of lipoprotein yolk globules and cortical alveoli in the cytoplasm due to phagocytosis (Fig. 4, E–G), was rare in the samples selected and was present in 3 of the 11 individuals for

² Witthames, P. R. 2009. Personal commun. Cent. Environ. Fish. Aquac. Sci., Lowestoft Lab., Pakefield Rd., Lowestoft, Suffolk NR33 0HT U.K.

³ Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.

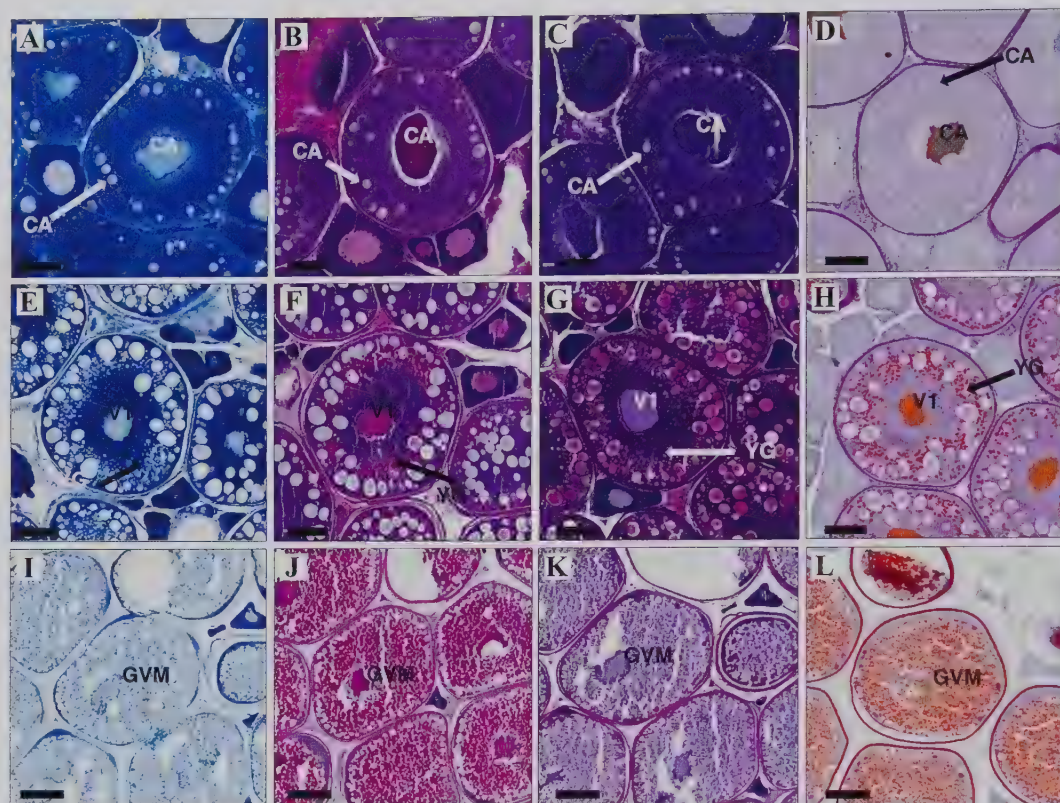


Figure 2

Histological images of 3 stages of oogenesis in gonad tissue from female Atlantic herring (*Clupea harengus*) collected in the Northwest Atlantic Ocean in 2015 and 2016: (A–D) oocytes in the cortical alveolar stage, with cortical alveoli (CA) around the periphery of each oocyte; (E–H) oocytes in early vitellogenesis (V1), with yolk granules (YG) partially filling the cytoplasm; and (I–L) oocytes in germinal vesicle migration (GVM). In each row, for comparison, images of tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. Labels without arrows refer to specific stages of oocyte development, and those with arrows refer to specific structures. The scale bars are equal to 60 μ m in panels A–D, 80 μ m in panels E–H, and 200 μ m in panels I–L. See Table 1 for a summary of sample sources and other collection information, and see Supplementary Table (online only) for additional details about each fish examined.

which slides were examined. Sections from 1 fish had multiple examples of beta atresia, which was recorded by all readers for all stains. In the other 2 fish, beta atresia was rare (usually a single instance annotated) and was recorded with a low level of confidence by 1 or 2 readers, for one or more stains (for PAS-MT or for H&E, PAS, and PAS-MT).

Haddock Across all 4 staining methods, oocytes in 7 of the 8 stages of oogenesis (all but ovulation) were visible in the slides for selected samples of haddock. Primary growth oocytes were present in every histology slide. Cortical alveoli marked the secondary growth stage of oogenesis and first appeared in the periphery of the cytoplasm as small, circular, white inclusions (Fig. 5, A–D). Early vitellogenesis followed the cortical alveolar stage, with the presence of lipoprotein yolk globules forming around the germinal vesicle (Fig. 5, E–H). As oocytes reached final

oocyte maturation, multiple stages of oogenesis (representing subsequent batches) were present in individual haddock (Fig. 5, I–L). The migration of the germinal vesicle toward the zona pellucida marked final oocyte maturation. Hydrated oocytes were also present and were identified as oocytes that remained within the follicle after the yolk globules completely fused together and water content was absorbed.

All 3 stages of POFs and both stages of follicular atresia were identified for haddock across all 4 methods of staining (Fig. 6). Recent POFs were irregular in shape, the theca and granulosa layers were distinctly visible, and the lumen was larger than a perinucleolar oocyte (Fig. 6, A–D). Intermediate POFs were slightly smaller, with a collapsed lumen (Fig. 6, E–H), and in slides made with PAS and PAS-MT stains, the contrast in colors between the theca and granulosa layers was more pronounced. Old POFs were smaller than a perinucleolar oocyte, with an absent

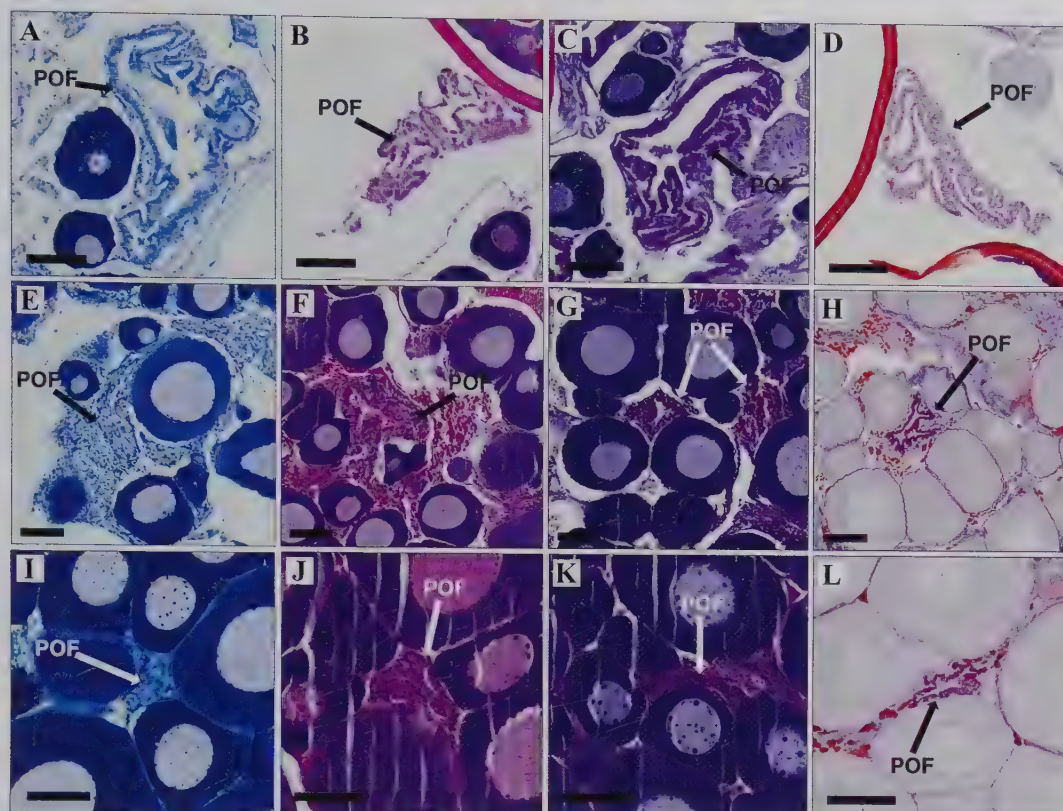


Figure 3

Histological images of 3 stages of postovulatory follicles (POFs) in gonad tissue from female Atlantic herring (*Clupea harengus*) collected in the Northwest Atlantic Ocean in 2015 and 2016: (A–D) recent, (E–H) intermediate, and (I–L) old. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 80 μ m in panels A–D and 60 μ m in panels E–L.

lumen and no distinction of the theca and granulosa layers (Fig. 6, I–L).

Follicular atresia was recorded as alpha or beta atresia (Fig. 7). Alpha atresia had a melted or liquefied appearance, with the zona pellucida breaking down but the contents within the cytoplasm sometimes still intact. Follicles in beta atresia were smaller in size than those with alpha atresia, and the internal components of the cytoplasm had been digested through phagocytosis.

Yellowtail flounder Across all 4 staining methods, oocytes in all 8 stages of oogenesis were identified in the slides for selected samples of yellowtail flounder. Primary growth oocytes were present in every histology slide. The cortical alveolar stage was identified with each stain used in this study, with oocytes slightly larger than a primary growth oocyte and faint circular, white inclusions near the periphery of the oocyte (Fig. 8, A–D). Early vitellogenesis was recorded when lipoprotein yolk globules began to appear as small dots close to the zona pellucida (Fig. 8, E–H). Similar to the haddock, the yellowtail flounder is a

batch-spawning species, and multiple oocyte stages were identified in fish with oocytes in the late vitellogenesis stage and the late maturation phase (Fig. 8, I–L). Late vitellogenesis was recorded when the lipoprotein yolk globules filled more than half of the cytoplasm, followed by the migration of the germinal vesicle. Germinal vesicle migration marked the final phase of oocyte maturation. In addition to germinal vesicle migration, hydrated oocytes still within the follicle were identified (Fig. 8, I–L). Ovulation was also observed in yellowtail flounder, with fully hydrated oocytes released from follicles and becoming eggs (Fig. 8, M–P).

All 3 stages of POFs were identified in yellowtail flounder across all 4 methods of staining (Fig. 9). Recent POFs were irregular in shape, giving them a wispy appearance, with a visible lumen and the theca distinguished from the granulosa layer in slides with most stains (Fig. 9, A–D). Intermediate POFs (Fig. 9, E–H) were widely seen and were more compact in size, with a collapsed lumen and thicker layers. Old POFs (Fig. 9, I–L) were completely degraded with no visible lumen, and in some cases, these

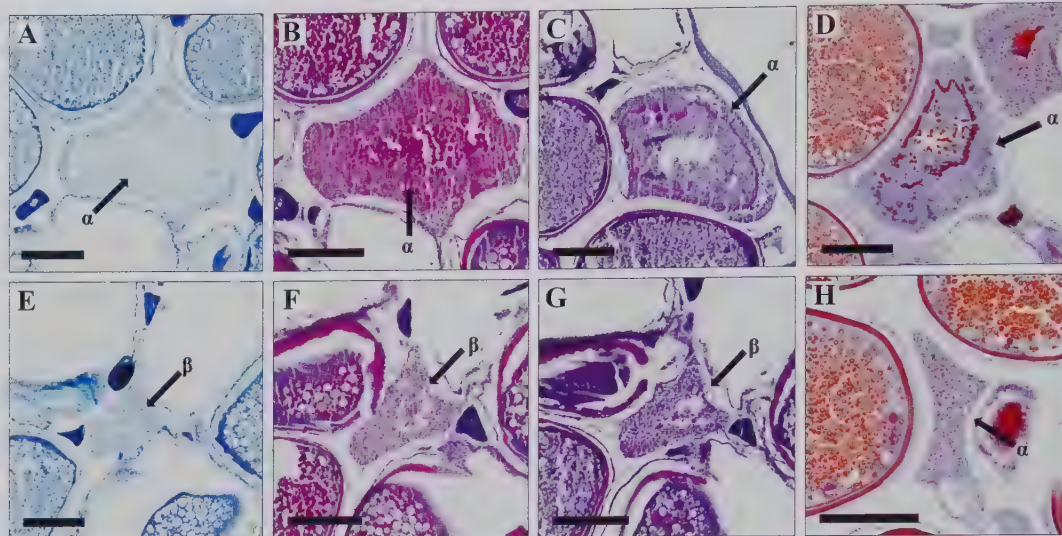


Figure 4

Histological images of 2 stages of follicular atresia in gonad tissue from female Atlantic herring (*Clupea harengus*) collected in the Northwest Atlantic Ocean in 2015 and 2016: (A–D) alpha (α), (E–G) beta (β), and (H) advanced (late) alpha. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. All scale bars are equal to 200 μ m.

POFs were more easily identifiable because they appeared darker in color (Fig. 9, K and L).

In addition, follicular atresia was also recorded for yellowtail across all 4 staining methods (Fig. 10). Alpha atresia was identified as having a melted or liquefied appearance, with the zona pellucida broken down and becoming discontinuous and irregular in thickness (Fig. 10, A–D). Beta atretic oocytes were more completely degraded, without any remaining yolk or zona pellucida, and they were smaller in appearance because the contents of the cytoplasm were digested through phagocytosis (Fig. 10, E–H).

Confidence levels for features

Oocyte development stages Confidence levels for identification of stages of oocyte development varied across all species for each staining method (Fig. 11). For each species, primary growth oocytes were easily distinguished with all 4 stains.

The cortical alveolar stage was the most difficult stage to consistently identify, and lack of certainty is evident for each species for all 4 stains. The PAS-MT staining method provided the highest confidence level for identification of cortical alveoli, in comparison to use of the TB stain, the method that had the lowest level of confidence. Similar results were found when using the H&E and PAS stains to identify oocytes with cortical alveoli. A closer look at the defining stage of cortical alveoli revealed a slight advantage in recognizing small, circular inclusions for the H&E stain over the PAS stain (Fig. 12).

Confidence levels in identifying the early vitellogenesis stage differed among the species and stains (Fig. 11). The highest confidence levels were found for the PAS-MT staining method. Use of the H&E and PAS stains resulted in similar, slightly lower confidence levels, and distinguishing lipoprotein yolk globules was most difficult with TB staining.

The stages of late vitellogenesis and germinal vesicle migration were easy to identify for all 3 species, with a very low percentage of uncertainty, when the TB and PAS stains were used (Fig. 11). For the identification of the stage of germinal vesicle breakdown, confidence levels were high for the PAS-MT and PAS staining methods, and there is some ambiguity in results from the use of the H&E stain. The lowest level of confidence in identifying germinal vesicle breakdown was found for TB staining.

Hydrated oocytes were easily identified with all stains. There was minor variation in confidence levels for distinguishing these oocytes, with high levels for the PAS and PAS-MT staining methods (Fig. 11). For both H&E and TB staining, levels of confidence were slightly lower but still medium to high.

The confidence levels for recognizing ovulation were highest for the PAS-MT staining method and lowest for the TB staining protocol, but this stage was only observed in yellowtail flounder (the samples from individuals of other species did not have oocytes in this stage) (Fig. 11).

Postovulatory follicles The confidence levels of readers in identifying and staging POFs ranged from high with

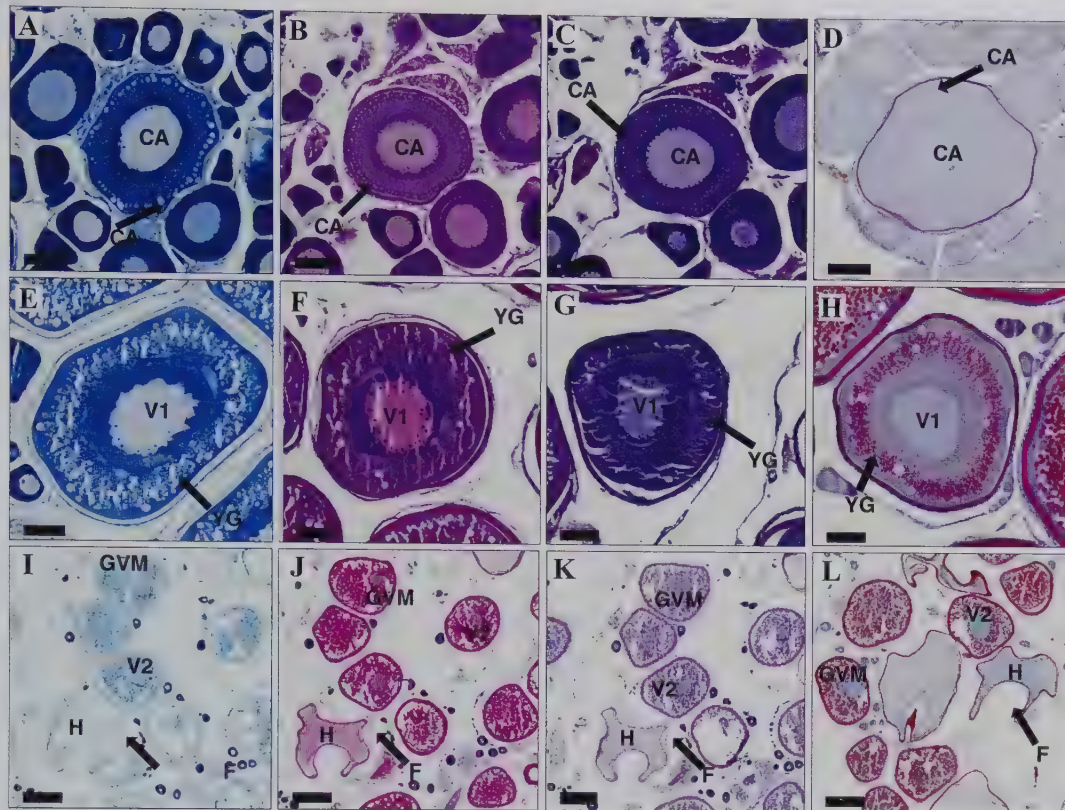


Figure 5

Histological images of 3 stages of oogenesis in gonad tissue from female haddock (*Melanogrammus aeglefinus*) collected in the Northwest Atlantic Ocean in 2013 and 2015: (A–D) oocytes in the cortical alveolar stage, with cortical alveoli (CA) around the periphery of the oocyte; (E–H) oocytes in early vitellogenesis (V1), with yolk granules (YG) partially filling the cytoplasm; and (I–L) oocytes representing multiple spawning batches—oocytes in late vitellogenesis (V2) and germinal vesicle migration (GVM) and a hydrated (H) oocyte within the follicle (F). In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. Labels without arrows refer to specific stages of oocyte development, and those with arrows refer to specific structures. The scale bars are equal to 60 μ m in panels A–H and 400 μ m in panels I–L. See Table 1 for a summary of sample sources and other collection information, and see Supplementary Table (online only) for additional details about each fish examined.

PAS-MT staining to low with TB staining (Fig. 13). Recent POFs were easily detectable for all species in slides with the PAS-MT stain and the least identifiable in slides with the TB stain. The H&E and PAS staining methods provided similar levels of confidence in identifying recent POFs, but results varied among species. For Atlantic herring, uncertainty in detection of recent POFs was greater with the PAS stain than with the H&E stain; however, the reverse occurred for haddock.

Intermediate POFs were most confidently identified by using the PAS-MT stain for all 3 species, with slightly lower certainty recorded by readers for haddock. As a POF collapses and becomes more compact and possibly confused with late-stage atresia, the concentric layers (theca and granulosa) of the follicle are brightly stained when using a PAS stain; therefore, in this study, use of the PAS

stain resulted in greater confidence levels for identifying intermediate POFs than use of the H&E stain. Intermediate POFs were hardest to recognize when the TB stain was used.

Old POFs are arguably the hardest POF stage to identify for all 3 species. When the PAS-MT and the TB stains were used, the highest and lowest levels of confidence were recorded, respectively. Results from the use of the H&E and PAS stains were similar, but when readers directly compared magnified images of an old POF (Fig. 14), they observed more detail in slides with the PAS stain than in those with H&E.

Atresia Both alpha and beta atresia were identified for all species and staining methods. When using the PAS-MT stain, readers had the highest certainty in recognizing

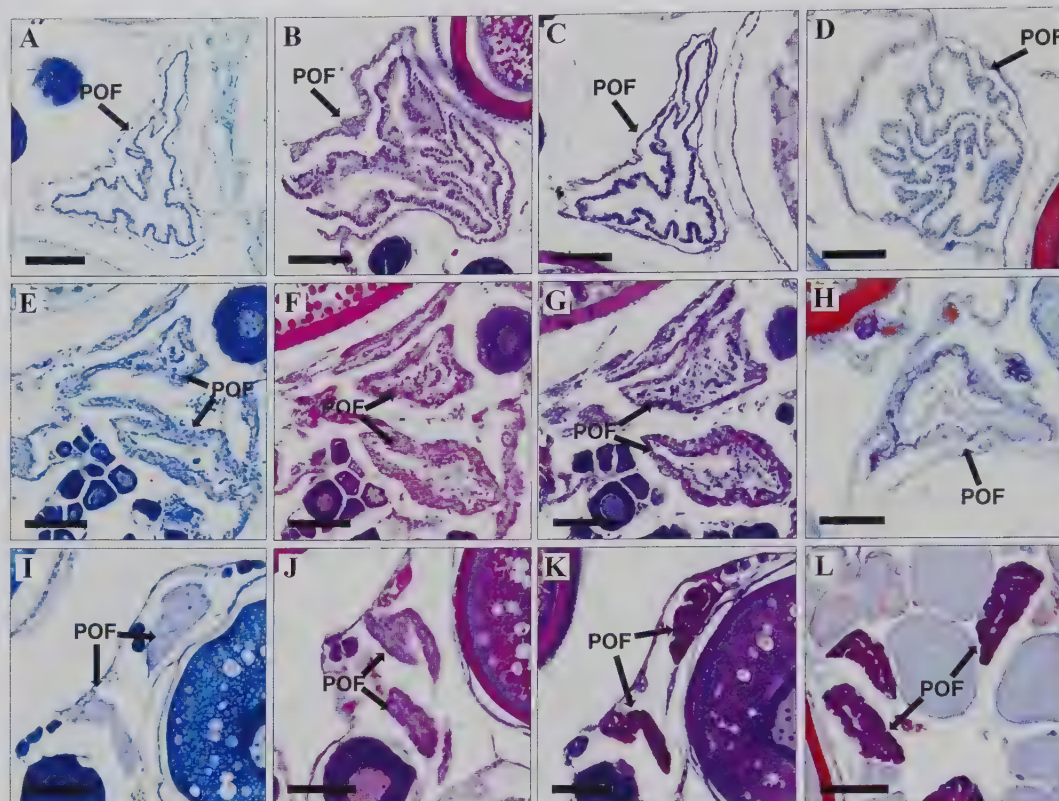


Figure 6

Histological images of 3 stages of postovulatory follicles (POFs) in gonad tissue from female haddock (*Melanogrammus aeglefinus*) collected in the Northwest Atlantic Ocean in 2013 and 2015: (A–D) recent, (E–H) intermediate, and (I–L) old. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 90 µm in panels A–D, 80 µm in panels E–H, and 60 µm in panels I–L.

alpha atresia for all 3 species, but identifying it with the TB stain proved to be difficult (Fig. 15). Use of the H&E and PAS stains resulted in similar confidence levels for recognition of alpha atresia. Beta atresia was a bit more difficult to identify because of the smaller size of oocytes in this stage and their similarity to an old POF. Readers were most confident in determining the presence of beta atresia when the PAS-MT stain was used, and beta atresia was much harder to confidently identify with the H&E, PAS, and TB stains.

Confidence levels across species

When all 3 species were compared, confidence levels across all 4 staining methods were the highest for yellowtail flounder, followed by Atlantic herring and then haddock (Fig. 11). For yellowtail flounder, a confidence level of nearly 100%, with minimal uncertainty, was found when the PAS-MT stain was used to identify the cortical alveolar stage. In slides with TB and PAS stains, identification of the cortical alveolar stage proved more difficult than

in slides with the H&E stain. Confidence levels also were low for readings of the TB slides in identifying the final 2 stages of oocyte maturation (germinal vesicle breakdown and hydration) and ovulation.

The PAS-MT staining method proved to be the best approach for identifying all stages of oogenesis in Atlantic herring. The overall confidence level for use of the H&E stain was slightly better than that for use of the PAS stain, and the most uncertainty occurred with the TB stain. For all 4 staining methods, readers had some hesitation in recognizing cortical alveoli in Atlantic herring. The highest level of confidence was found for use of the PAS-MT stain, followed by use of the H&E stain. With the PAS and TB stains, identification of cortical alveoli in oocytes was equally as difficult. Readers had slight uncertainty in distinguishing germinal vesicle migration when using the PAS and TB stains, and they lacked some confidence in identifying hydrated oocytes when using the H&E and TB stains.

The greatest difference in confidence levels across staining methods occurred for haddock. Use of the PAS-MT and

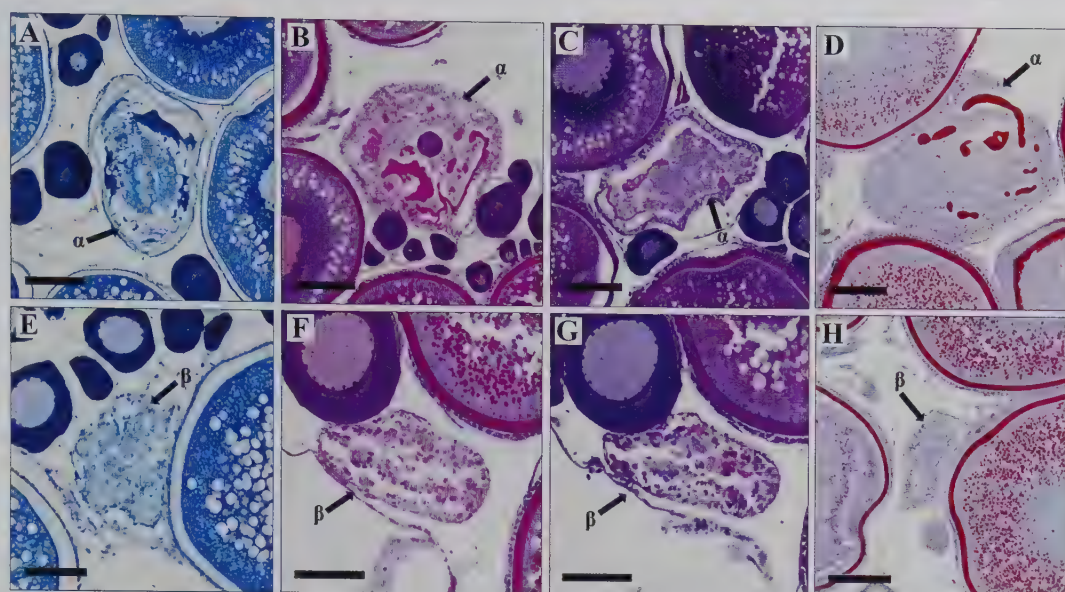


Figure 7

Histological images of 2 stages of follicular atresia in gonad tissue from female haddock (*Melanogrammus aeglefinus*) collected in the Northwest Atlantic Ocean in 2013 and 2015: (A–D) alpha (α) and (E–H) beta (β). In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 100 μ m in panels A–D and 60 μ m in panels E–H.

H&E stains resulted in higher confidence levels for identifying oocyte development stages than use of the other stains. For the cortical alveolar stage, the certainty of readers was slightly better with the PAS-MT stain than with the H&E stain. Distinguishing cortical alveoli in oocytes was difficult with both the PAS and TB stains. With all stains, readers had some uncertainty in recognizing early vitellogenesis, with the PAS-MT staining method being the best for identification of early lipoprotein yolk globules and TB staining being the technique with which it was most difficult. Germinal vesicle breakdown was sometimes difficult to consistently identify by using the PAS and TB stains.

Discussion

In this study, we took a comprehensive look at histological staining methods, comparing their effectiveness for identification of different stages of oogenesis and related gonad characteristics of fish across 3 phylogenetically different species. Although the same features should have been observed in slides with different stains for each individual, we found differences among stains in the accuracy in identifying certain structures. Studies in which tissue staining techniques have been compared by using samples for different individuals are useful (e.g., Alonso-Fernández et al., 2011); however, such assessments are less powerful than

comparisons made by using samples from the same individuals, as was done in our study (and in Lowerre-Barbieri et al., 2023) to remove individual variation. Herein, we discuss the advantages and disadvantages of the staining methods we evaluated, highlight the potential implications of the different levels of information and confidence obtained by using these methods for fish reproduction studies, and suggest best practices for gonad histology of fish.

Staining methods

Ultimately, the method involving the most complex stain, PAS-MT, proved to be the best for confident identification of all stages of oogenesis, including early maturation of oocytes, postspawning indicators (POFs and thick tunica), and atresia. Although we acknowledge that the true development stages were not known, we equate high confidence levels with high precision (repeatability of measures) and high accuracy (reduced bias). Readers had more experience with the PAS-MT stain, but the higher confidence they had in using that stain reflects the greater detail they observed for specific features and structures and was not simply due to familiarity. A disadvantage of this complex stain, aside from its high cost, is that the pararosaniline in the PAS-MT formula is a carcinogen and requires special handling (Coghill, 1996).

The contrasting colors of the PAS-MT stain highlight important cellular features, in a way that those of the

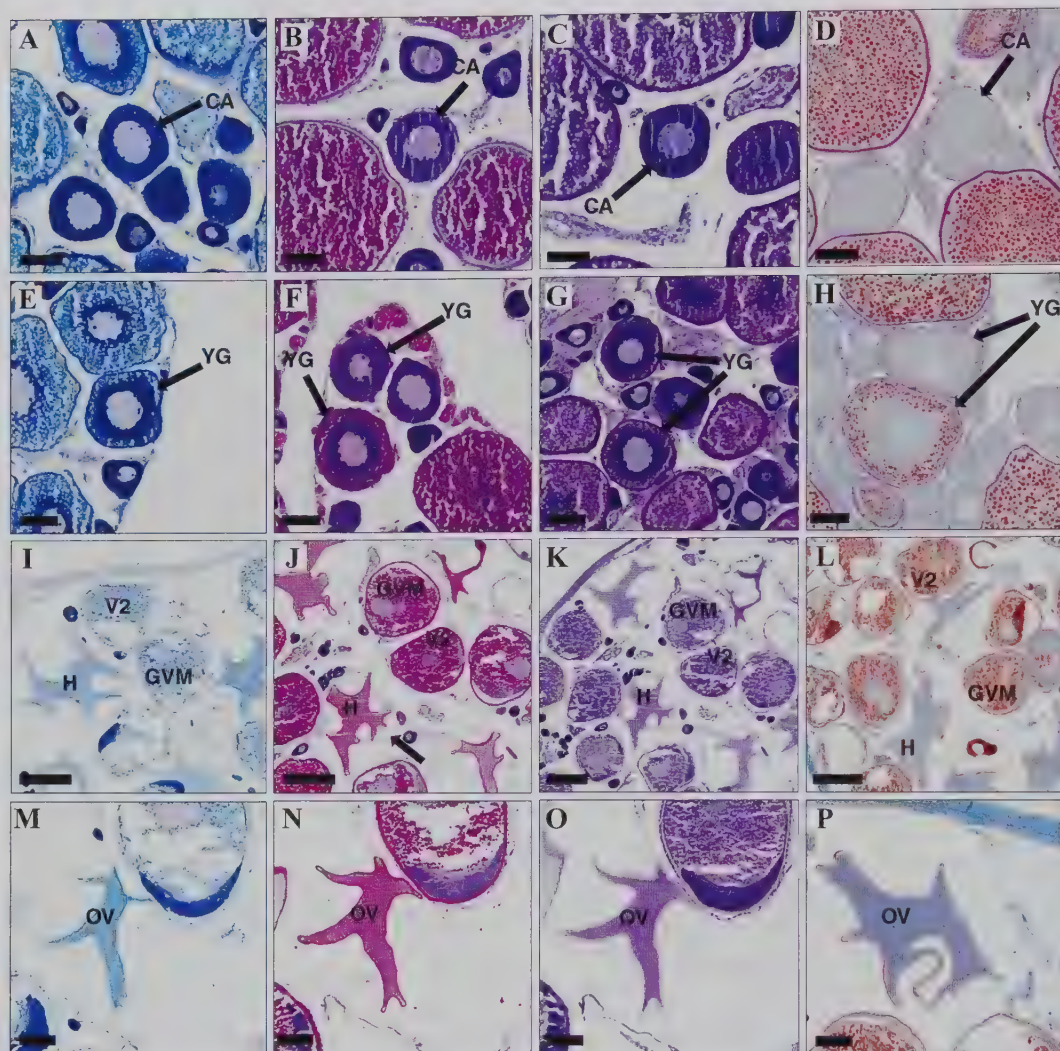


Figure 8

Histological images of 4 stages of oogenesis in gonad tissue from female yellowtail flounder (*Myxopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2012, 2018, and 2019: (A–D) oocytes in the cortical alveolar stage, with cortical alveoli (CA) around the periphery of the oocyte; (E–H) oocytes in early vitellogenesis (V1), with yolk granules (YG) partially filling the cytoplasm; (I–L) oocytes representing multiple spawning batches—oocytes in late vitellogenesis (V2) and germinal vesicle migration (GVM) and hydrated (H) oocytes within the follicle (F); and (M–P) ovulated (OV) eggs. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. Labels without arrows refer to specific stages of oocyte development, and those with arrows refer to specific structures. The scale bars are equal to 60 μ m in panels A–H, 300 μ m in panels I–L, and 100 μ m in panels M–P. See Table 1 for a summary of sample sources and other collection information, and see Supplementary Table (online only) for additional details about each fish examined.

3 simpler, less expensive, and more commonly used stains—TB, H&E, and PAS—do not. Cytoplasm has a lighter color with the PAS-MT stain, allowing cortical alveoli and lipoprotein yolk globules to stand out. The presence of cortical alveoli is used to identify when a female has crossed a key physiological threshold of hormone and gonad development (Lowerre-Barbieri et al.,

2023) toward maturation, even though some species may take several months or years to attain functional maturity (Kennedy et al., 2011b; Press et al., 2014; McBride et al., 2022b). In samples from fish that had spawned, confidence levels in identifying all stages of POFs were highest when the PAS-MT stain was used. As a POF degrades, the deep-purple color of this stain remains in the follicle layers,

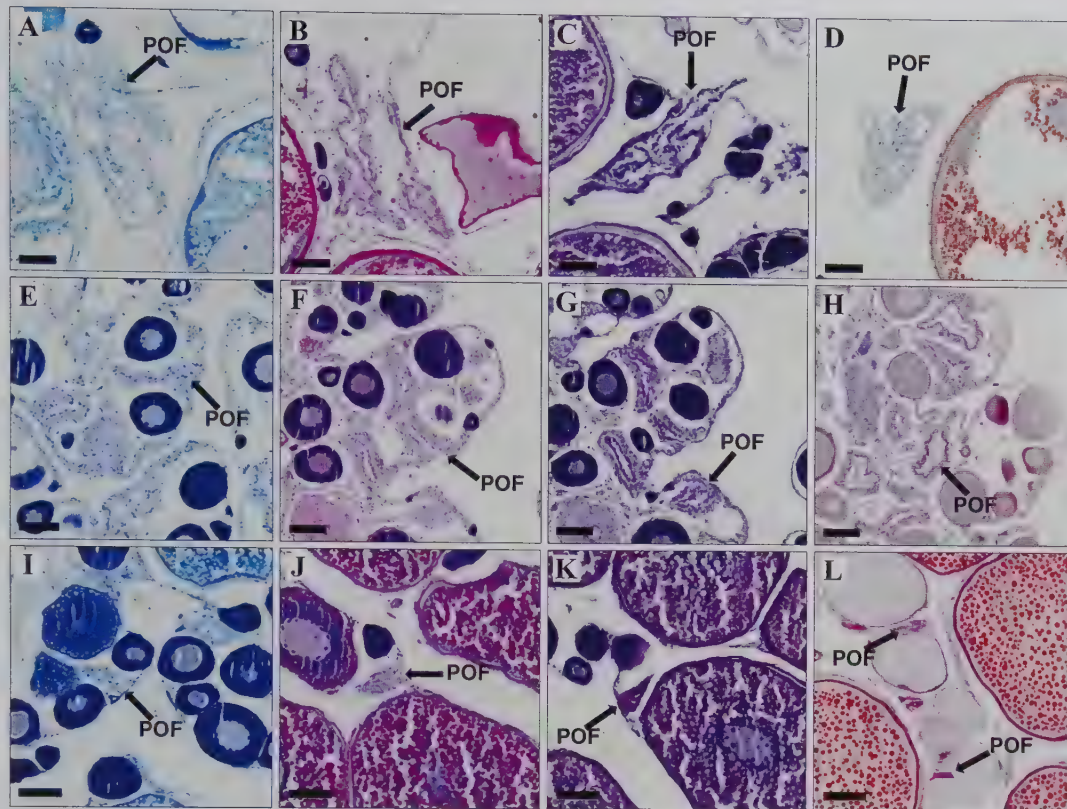


Figure 9

Histological images of 3 stages of postovulatory follicles (POFs) in gonad tissue from female yellowtail flounder (*Myxopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2012, 2018, and 2019: (A–D) recent, (E–H) intermediate, and (I–L) old. In each row, for comparison, images of adjacent tissue sections from the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. All scale bars are equal to 60 μm .

making the small structures easy to pick out. In addition, the deep-purple color also helps to differentiate between an oocyte in a very late atretic phase and a very old POF. Both alpha and beta atresia stood out and were easier to identify with the PAS-MT stain.

Use of the simple monochromatic TB stain resulted in the lowest confidence in identifying structures for all 3 readers. Readers had a high degree of uncertainty when distinguishing stages of oocyte development, particularly the secondary growth stages. In the secondary growth stages, when cortical alveoli and lipoprotein yolk globules first appeared, these features were difficult to identify with confidence because of the subtle contrast in stain intensity throughout sections in slides. In addition, the presence of POFs and their stages were difficult to distinguish with this monochromatic stain. When identifying old POFs, readers almost never recorded their presence with a high confidence level. Similarly, when beta atresia was observed, it was rarely recorded with a high level of confidence. Importantly, low confidence levels in identification of old POFs (Fig. 14) and beta atresia (Figs. 4, 7,

and 10) can lead to uncertainty in assignments of maturity stage that rely on information about these features. With the TB stain, both of these structures can look very similar; however, the presence of old POFs is a definite sign of spawning activity, and beta atresia is not.

Although we refer to it as a single stain, toluidine blue has metachromatic properties that make it well suited for a range of applications (Sridharan and Shankar, 2012; Longenecker and Langston, 2018; Vidal and Mello, 2019; Longenecker et al.¹). Schemmel and Friedlander (2017) successfully identified oocyte and POF stages using a TB stain in histological characterization of the reproductive state of convict surgeonfish (*Acanthurus triostegus*). Kjesbu et al. (2011) used TB alone to study the shift of primary growth oocytes to cortical alveolar and vitellogenic stages. They revealed a seasonal progression of cytoplasmic features, such as a circumnuclear ring, cortical alveoli, 2 stages of vitellogenesis, and POFs in laboratory-reared Atlantic cod (Kjesbu et al., 2011, fig. 6). Familiarity with this species and the routine nature of sampling at specific times of year allowed

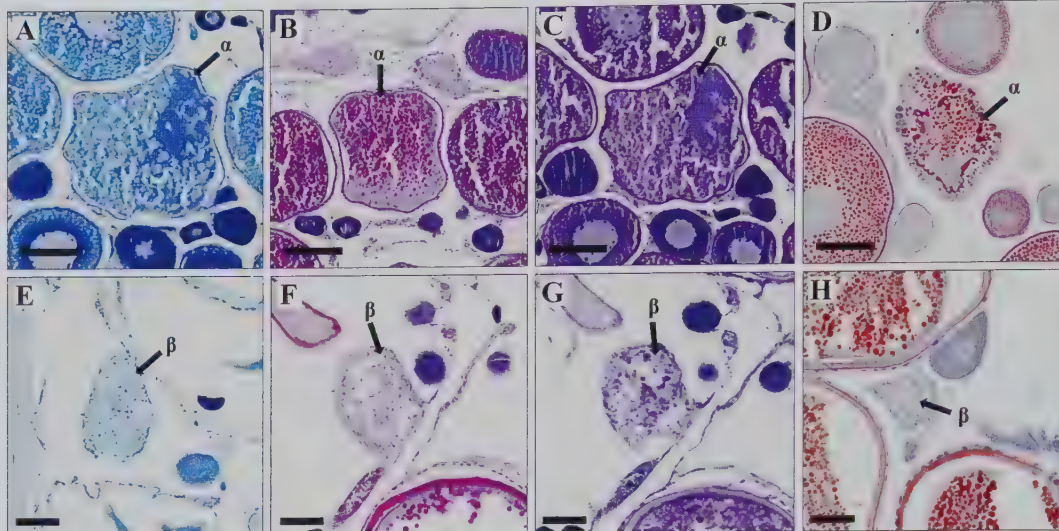


Figure 10

Histological images of 2 stages of follicular atresia in gonad tissue from female yellowtail flounder (*Myxopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2012, 2018, and 2019: (A–D) alpha (α) and (E–H) beta (β). In each row, for comparison, images of adjacent tissue sections of the same individual are shown, each with a different stain used (from left to right): toluidine blue, hematoxylin and eosin, periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome. The scale bars are equal to 100 μ m in panels A–D and 60 μ m in panels E–H.

Kjesbu⁴ and his collaborators to use the inexpensive TB stain for a variety of purposes: to measure the size of the “leading cohort” of oocytes in a gonad (Anderson et al., 2020), to identify individual Atlantic cod for the presence of atresia (Witthames et al., 2013), or to check for POFs to screen for the suitability of an individual female for estimation of fecundity.

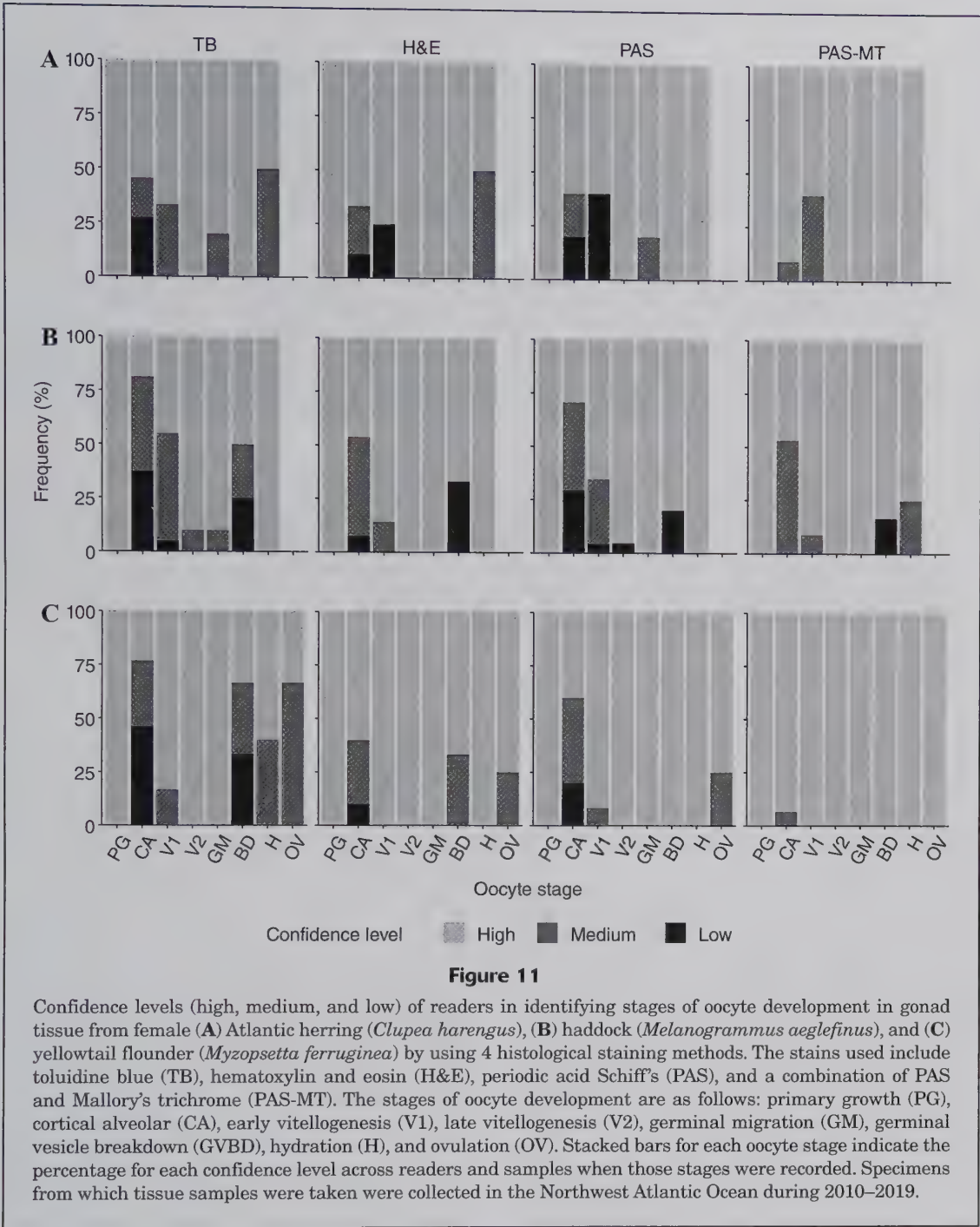
Use of the H&E and PAS stains resulted in similar levels of confidence in identification of features. Confidence levels in distinguishing oocyte development stages were higher with the H&E stain than for the TB stain but were still below the levels for oocyte staging with the PAS-MT stain. Interestingly, for differentiation between 2 early stages, cortical alveoli and early vitellogenesis, readers had slightly more confidence when using the H&E stain than when using the other stains. Cytoplasm has a light pinkish color when the H&E stain is applied but a dark-purple color when the PAS stain is used. The lighter hue of cytoplasm with the H&E stain makes cortical alveoli and lipoprotein yolk globules stand out more than they do with the PAS stain. For all of the 3 species evaluated, subtle differences in oocyte development were evident, leading to uneven ranking of staining methods across species.

Use of the H&E and PAS stains produced different results for identification of POFs. Readers had more confidence in identifying the presence and determining the

stage of POFs with the PAS stain than with the H&E stain. Recent POFs were dark purple in slides with the PAS stain and light pink in slides with the H&E stain. As POFs progressed from the recent stage to the intermediate and old stages, little change in coloration occurred with H&E staining. In contrast, as POFs degraded, greater intensity and affinity of pigment was observed with the PAS stain, making them more obvious, and readers were able to identify intermediate and old POFs more easily. The dark-purple color remained as a POF broke down further, in addition to a magenta color becoming more noticeable in the granulosa layer. This magenta-like appearance remained in old POFs, making it possible to distinguish reliably between an old POF and very old atresia.

Among a plethora of staining methods we did not consider, some techniques are notable for their use in histological studies of fish gonads. Quintero-Hunter et al. (1991) modified a PAS stain with Weigert's iron-hematoxylin as a nuclear stain and metanil yellow as a counterstain. The PAS part of this stain highlights melano-macrophage centers in a bright pink hue, in contrast to the dull-brown centers (“brown bodies”) that have been observed with H&E (McBride et al., 2002, 2012), and it reveals the PAS-positive nature of the yolk in certain fish orders (e.g., Beloniformes; McBride and Thurman, 2003). Stemming from an interest in the transition from oogonia to oocytes that occurs along basement membranes (i.e., folliculogenesis), Grier (2012, 2016) omitted the hematoxylin to better reveal basement membranes, and Grier et al. (2018) used

⁴ Kjesbu, O. S. 2024. Personal commun. Inst. Mar. Res., P.O. Box 1870 Nordnes, 5817 Bergen, Norway.



a reticulin stain described by Mazzoni et al. (2014) to high-light basement membranes. Examples of the use of these stains and 2 others, alcian-blue as well as thionin and osmium, were depicted by Grier et al. (2016). Staining with Prenant–Gabe’s trichrome involves application of a fast green stain after H&E, highlighting connective tissues, including the gonad wall as a light green, and it has recently been applied in histological studies of several fishes (Sauger et al., 2020; Dubroca et al., 2023; Heude-Berthelin et al., 2024).

Implications for reproduction studies

With a large array of stains to choose from (Riva et al., 2014) and each providing different levels of accuracy and precision for specific features, the optimal stain for any given study will differ depending on the objectives. As described herein, both oocyte development and spawning patterns differ across species. Analyzing and interpreting gonad histology can be complex and require identification of specific microstructural features; therefore, the choice of

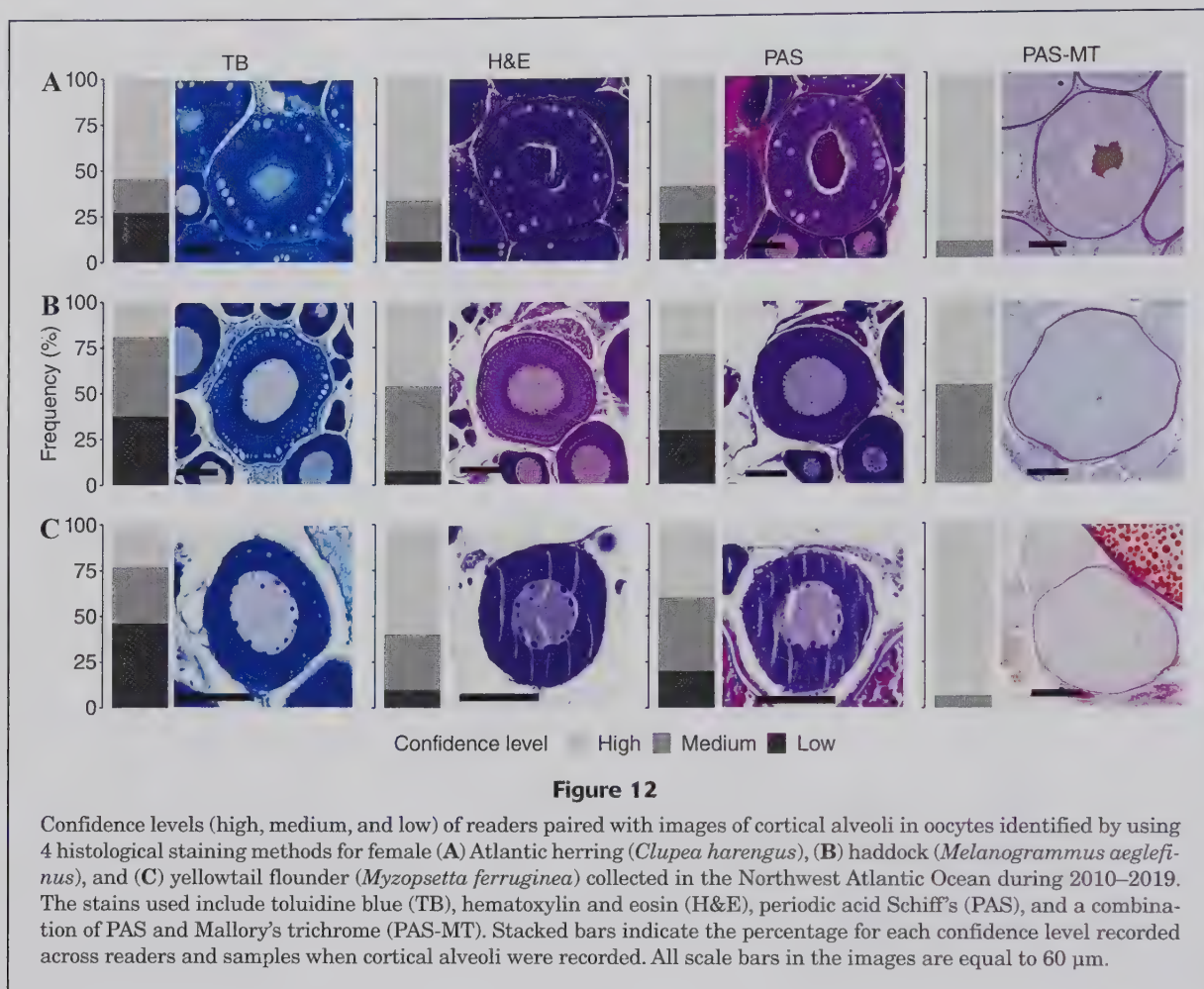


Figure 12

Confidence levels (high, medium, and low) of readers paired with images of cortical alveoli in oocytes identified by using 4 histological staining methods for female (A) Atlantic herring (*Clupea harengus*), (B) haddock (*Melanogrammus aeglefinus*), and (C) yellowtail flounder (*Myxopsetta ferruginea*) collected in the Northwest Atlantic Ocean during 2010–2019. The stains used include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Stacked bars indicate the percentage for each confidence level recorded across readers and samples when cortical alveoli were recorded. All scale bars in the images are equal to 60 μ m.

stain used in a study can have a large effect on the interpretation of (and level of confidence in) information obtained through gonad histology.

In fish reproduction studies, gonad histology is used to evaluate a variety of properties, including maturity, spawning seasonality, spawning frequency, and fecundity (e.g., atretic downregulation). The cellular features required to accurately assess each of these properties will vary, as will stain selection, consequently. In this section, for each of the reproductive properties typically evaluated in studies of fish, we expand on the advantages and disadvantages of various staining methods across the species evaluated in this study and in other studies.

Information on maturity plays an important role in managing sustainable fisheries. The size and age at maturity mark the critical point of transition when an individual becomes capable of reproducing, a transition occurring only once in an individual's lifetime that is most often defined in terms of physiological maturity. In other words, maturity is assumed once the critical developmental threshold of the brain-pituitary-gonad axis that regulates hormonal and gonadal development is passed (Brown-Peterson et al., 2011; Lowerre-Barbieri et al., 2023). However, recently, estimation of functional maturity, when

fish are spawning or are going to spawn in the current or upcoming spawning season, is of greatest relevance as input to a fish stock assessment model, rather than when a fish passes some hormonal threshold (Prince et al., 2022). Moreover, in some species, individuals may initiate but abort a clutch of vitellogenic oocytes, thereby delaying functional maturity and misleading estimation of maturity (Kennedy et al., 2011a; Conrath, 2017; Lefebvre et al., 2018; McBride et al., 2022b). Although maturity classification can sometimes be fairly straightforward with simple macroscopic evaluations, accurate identification of functionally mature individuals outside of peak spawning periods may require the use of gonad histology.

Histology, regardless of the specific staining method used, provides several significant advantages over macroscopic methods, including an increased level of detail, sub-cellular stain affinities that aid identification, a means to assess tunica thickness, and a physical archival record that can be reevaluated. For these reasons, histology-based maturity estimates are often assumed to be more accurate than macroscopic determinations, without consideration of the nuances and potential biases of using different stains in identification of key features. For example, given that an individual with oocytes in the cortical

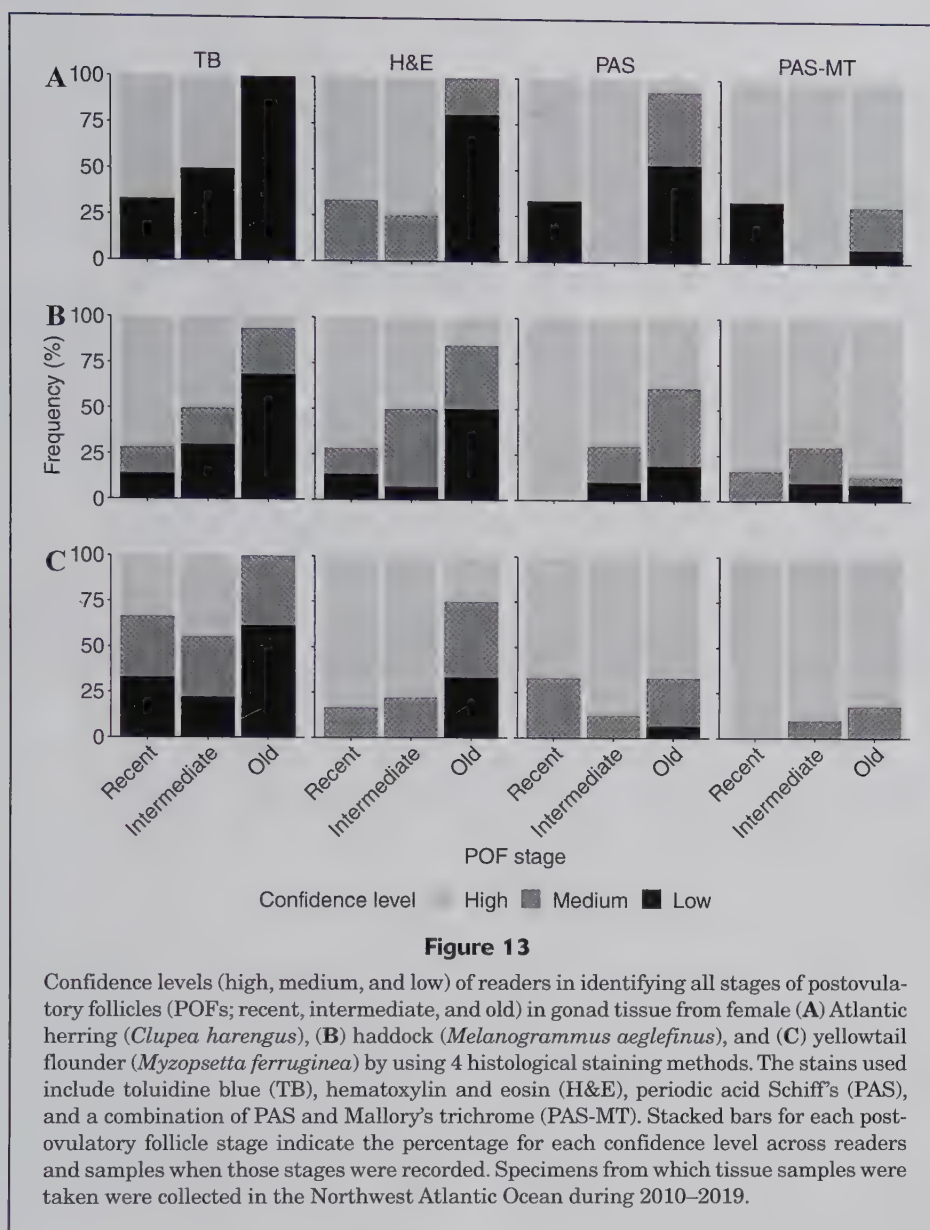


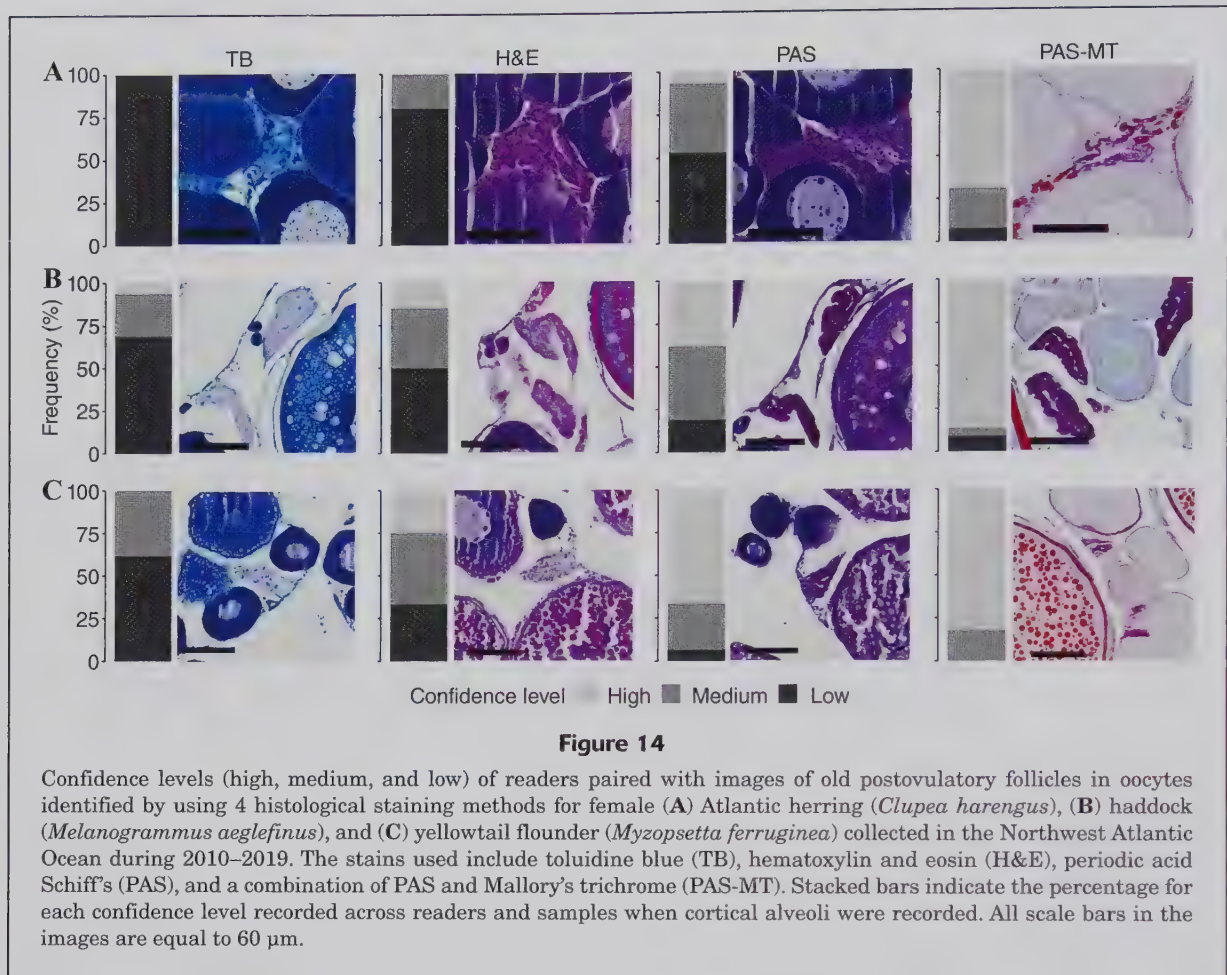
Figure 13

Confidence levels (high, medium, and low) of readers in identifying all stages of postovulatory follicles (POFs; recent, intermediate, and old) in gonad tissue from female (A) Atlantic herring (*Clupea harengus*), (B) haddock (*Melanogrammus aeglefinus*), and (C) yellowtail flounder (*Myxopsetta ferruginea*) by using 4 histological staining methods. The stains used include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Stacked bars for each post-ovulatory follicle stage indicate the percentage for each confidence level across readers and samples when those stages were recorded. Specimens from which tissue samples were taken were collected in the Northwest Atlantic Ocean during 2010–2019.

alveolar stage at a certain time of year may be considered mature, using a stain with which the presence of cortical alveoli is ambiguous can increase the risk of misidentifying the stage of maturity. For another case, consider that the accurate classification of skipped spawners may require successful identification of both tunica thickness and old POFs. Tunica thickness may be equally interpretable by using any number of stains, but old POFs, which may last more than 1 year in some cold-water species, may not be evident with all stains (e.g., Rideout et al., 2005, Skjæraasen et al., 2009, McBride et al., 2013, Press et al., 2014). Such false negatives (not seeing POFs because of the stain used) may bias estimates of spawning frequency. In most warm-water fishes, POFs degrade much faster than those in cold-water species and are rarely visible 48 h after spawning. Therefore, because old POFs do not

persist long enough in warm waters to serve as seasonal markers of spawning, the use of special stains for distinguishing them is not warranted for these species.

In contrast to the onset of maturity, which occurs only once, annual patterns of oocyte development that repeat many times over a reproductive lifetime are useful in determining the optimal seasons for identification of spawning contingent, spawning frequency, fecundity, skipped spawning, and energetics and condition of fish before, during, and after spawning. For some species, components of a population spawn at different times of year (e.g., Atlantic herring; Wuenschel and Deroba, 2019), and oocyte development stage at a given time of year can be used to differentiate such contingents. We found little difference among staining methods in confidence level for distinguishing vitellogenic and mature



oocytes, indicating that all stains would perform equally in studies with objectives to relate other factors (e.g., feeding, location, and behavior) to these stages of oocyte development.

Microstructural features may also be used to identify fish that are actively spawning, an important component of defining the spawning fraction (or frequency) for estimation of indeterminate fecundity (McBride et al., 2012). Features that are used as markers for active spawning can be used to determine spawning locations (McBride et al., 2003; Lowerre-Barbieri et al., 2009) and to improve understanding of spawning behaviors, such as diel reproductive periodicity (McBride et al., 2002, 2003; Hyle et al., 2014). These features typically include oocytes in germinal vesicle breakdown, hydration, and ovulation and recent POFs; therefore, reliance on histological staining methods that bias the identification of these stages can lead to bias in estimates of spawning fraction or spawning areas. Although we found similar levels of confidence in identifying recent POFs across the 4 methods we evaluated, confidence levels declined for distinguishing old POFs with the TB and H&E stains.

Accurate identification of recent POFs is also a requirement when determining fecundity of group-synchronous batch spawners because their absence indicates that no

batches were released yet that season (Witthames et al., 2009; McElroy et al., 2016). Because cold-water species have persistent POFs, studies of their reproduction require accurate identification of only recent POFs. As a result, stain selection will have little effect on studies of the fecundity of cold-water species. In contrast, the potential biases in detection of certain stages for a selected stain must be evaluated for histological studies that require accurate estimation of multiple stages of POFs, as is necessary for assessing indeterminate fecundity of warm-water fishes (Ganias et al., 2014).

Our level of confidence in identifying atresia (both alpha and beta) was variable across the species and staining methods evaluated. Accurate identification of atresia is important in fecundity studies, and biases could affect fecundity estimates in several ways. In stereological fecundity methods (Emerson et al., 1990; Medina et al., 2002; Aragón et al., 2010; Ganias et al., 2014), bias in detection of alpha atresia (including that due to handling and storage; Schemmel and Brown-Peterson, 2023) will affect what are counted as viable oocytes and, therefore, included in fecundity estimates. Additionally, studies in which atresia is quantified to refine fecundity estimates or determine the timing and rates of fecundity downregulation require accurate identification of both phases of

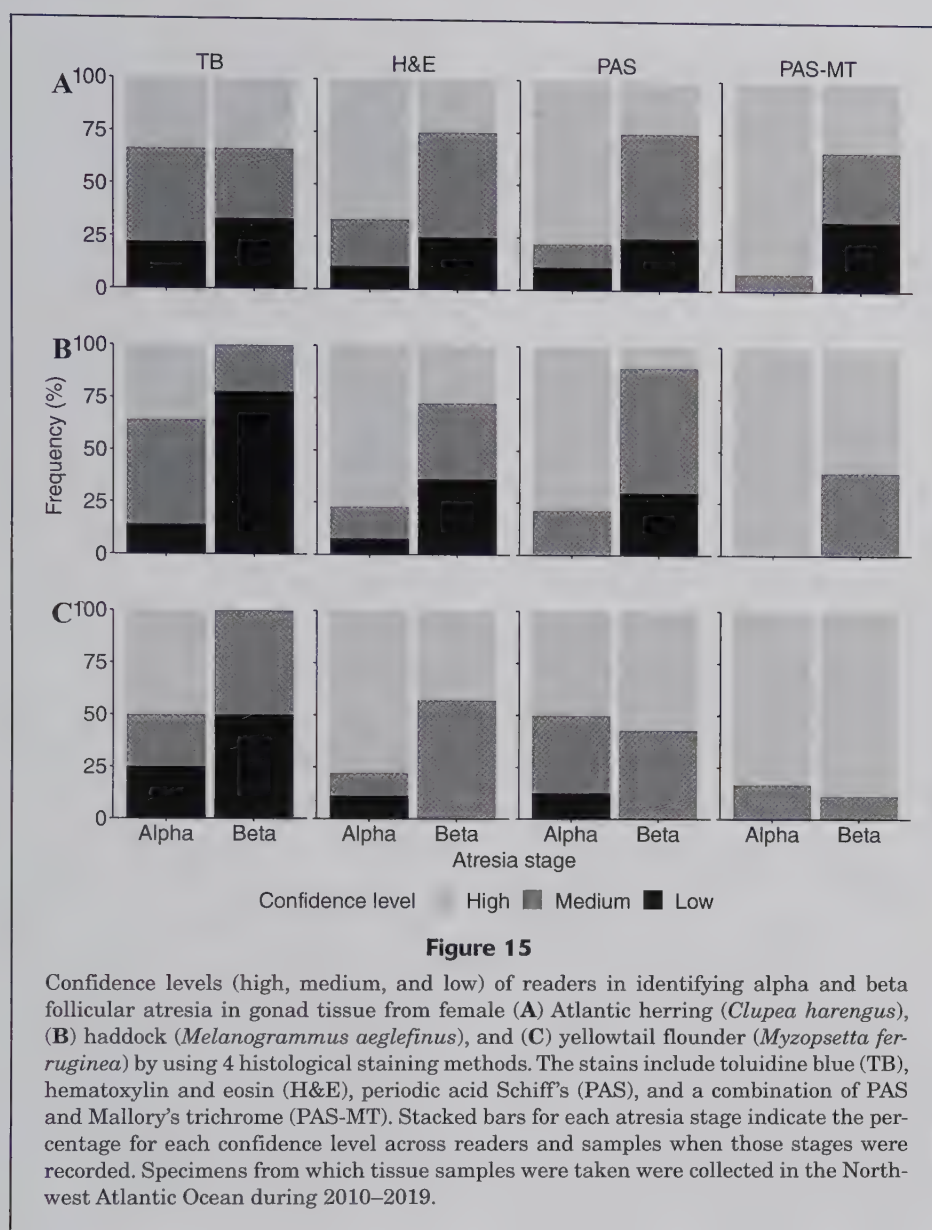


Figure 15

Confidence levels (high, medium, and low) of readers in identifying alpha and beta follicular atresia in gonad tissue from female (A) Atlantic herring (*Clupea harengus*), (B) haddock (*Melanogrammus aeglefinus*), and (C) yellowtail flounder (*Myxopsetta ferruginea*) by using 4 histological staining methods. The stains include toluidine blue (TB), hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), and a combination of PAS and Mallory's trichrome (PAS-MT). Stacked bars for each atresia stage indicate the percentage for each confidence level across readers and samples when those stages were recorded. Specimens from which tissue samples were taken were collected in the North-west Atlantic Ocean during 2010–2019.

atresia (Andersen⁵; Kjesbu et al., 2010; McElroy et al., 2016). We documented variable confidence levels for identification of atresia across staining methods, and such variation could bias estimation of atresia, downregulation, and fecundity. On the basis of these results, investigators intending to quantify atresia should carefully consider which staining method they plan to use.

Although the sequence of oocyte development, from oogenesis to mature egg, is similar across most fishes, the pace of oogenesis varies widely across species. Therefore, efforts to relate specific stages or thresholds of oocyte development to feeding, condition, or environmental factors will require accurate and unbiased determination of oocyte stages. The rate of oocyte development through specific stages (e.g., the cortical alveolar stage) may be fleeting in some warm-water fishes, and adjacent stages (e.g., early vitellogenesis) that are more easily identified with a given stain may be used to obtain an unbiased estimate of reproductive state. However, in cases in which oocyte development is protracted (as it is in many cold-water species) with stages (e.g., the cortical alveolar stage) that are difficult to identify persisting for long periods before a fish transitions to later stages that are easier to identify (e.g., early

⁵ Andersen, T. E. 2003. Unbiased stereological estimation of cell numbers and volume fractions: the disector and the principles of point counting. In Report of the working group on modern approaches to assess maturity and fecundity of warm- and cold-water fish and squids, Bergen, Norway, 4–7 September 2001 (O. S. Kjesbu, J. R. Hunter, and P. R. Witthames, eds.), p. 11–18. Inst. Mar. Res. and Res. Council Norway, Bergen, Norway. [Available from website.]

vitellogenesis), such an approach may bias interpretations. Potential biases and misclassifications are not equally distributed across stages of oocyte development because the pace of oogenesis is highly variable across species (specific stages are not present for similar lengths of time in different species). Therefore, investigators will need to consider whether the specific oocyte development stages of interest warrant the more accurate classification that is possible by using special staining.

Conclusions

In practice, selection of histological stain must balance the benefits and drawbacks with the cost of identifying or misidentifying specific structures or stage of oocyte development discussed herein. It is not surprising that the advantages and disadvantages of using different staining methods vary depending on the objectives of a study. A single one-size-fits-all approach (e.g., one in which H&E is the only stain used) may provide logistical and pricing advantages but sacrifice precision or accuracy in the identification of certain features. In our review of recent literature, H&E was by far the most widely used stain. Standardization to H&E affords advantages in practice, such as automated slide processing, stable shelf life of solutions, lower toxicity than alternatives, and a simple protocol. Given that most fish biologists lack formal training or experience in histological techniques, there is likely a tendency to adopt H&E as the standard stain. Although we did not find a broad level of bias when using H&E for most applications, other staining methods provide greater accuracy and precision for identification of some important features. For example, PAS and PAS-MT staining can provide greater detail and accuracy in determining presence of cortical alveoli and late stage POFs, both of which can exist for long periods of time in cold-water species and are important markers for physiological and functional maturity. Researchers should consider using alternative staining methods to evaluate false positive and false negative detections of specific diagnostic features when their objectives require it, and we encourage experimentation with new techniques when appropriate.

Resumen

La histología de las gónadas complementa la investigación sobre la historia de vida de las especies de peces y proporciona mayor exactitud y precisión que la caracterización macroscópica de la gónada para determinar los patrones de oogénesis a nivel celular y de madurez a nivel individual. En pesquerías, la histología mejora el estimado de la biomasa de adultos y reproductores de la población, la identificación de la estacionalidad y sus zonas de desove, y la preselección de especímenes para el cálculo de la fecundidad anual. Sin embargo, en la mayoría de los estudios se han utilizado un único método de tinción (hematoxilina y

eosina). En este estudio sobre 3 especies taxonómicamente diversas, utilizando 4 métodos de tinción diferentes de complejidad variable (grado de contratinción), comparamos los niveles de confianza en la identificación de 8 etapas de la oogénesis, la presencia y el nivel de degradación de los folículos postovulatorios y las células germinales atrésicas (vitelogénicas). Como se esperaba, el método de tinción monocromática menos costoso proporcionó el nivel de confianza más bajo, mientras que el método de contratinción más costoso y complejo proporcionó el nivel de confianza más alto, con la tinción de hematoxilina y eosina y otro método de contratinción simple entre ambos. El efecto del método de tinción fue más evidente en la identificación de los alvéolos corticales, que puede afectar a la estimación de la talla o la edad de madurez, y en la identificación de los folículos postovulatorios, que puede afectar a la estimación de la frecuencia de desove. Estos resultados son ampliamente aplicables para determinar las mejores prácticas.

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Abstract—Black sea bass (*Centropristis striata*) feed on epibenthic prey, particularly decapods, and become more piscivorous as they grow larger, a trend that has energetic consequences for growth. In the coming decades, construction of wind energy systems will expose black sea bass to a large-scale manipulation of continental shelf habitats in the northeastern United States. In this study, we tested the effects of habitat type (natural versus artificial reef habitats) on size, age, growth, diet, and trophic dynamics of black sea bass, using 407 specimens collected with rod-and-reel gear near Ocean City, Maryland, in 2016 and 2018. No significant differences in fish length, age, size at age, or diet are attributable to habitat. The stable isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in samples of 3 tissue types (liver, muscle, and mucus) vary significantly by habitat except for $\delta^{15}\text{N}$ values in mucus samples. Across tissue type, $\delta^{13}\text{C}$ values are higher for fish at artificial habitats, indicating more littoral than pelagic feeding. We conclude that, although both types of reef habitats are ecologically equivalent as a source of growth and diet for black sea bass, the differences in stable isotope signatures indicate functional differences in system energy flow that should be investigated further in this and other marine species that will be affected by offshore wind energy development.

Comparing the localized feeding ecology of black sea bass (*Centropristis striata*) at natural and artificial reefs: gut content and stable isotope analyses

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In the coming decades, black sea bass (*Centropristis striata*) will be exposed to a large-scale manipulation of the ecosystem of the U.S. continental shelf, with construction of wind energy systems that will significantly increase the proportion of structured habitats along the coasts of Maryland and New England (Kimmell and Stalenhof, 2011; Methratta et al., 2020). The black sea bass is a reef-oriented fish species (Steimle et al., 1999; Steimle and Zetlin, 2000; Fabrizio et al., 2013, 2014), and it may or may not benefit from more structured habitats in this region, which overlaps with the distribution of its northern stock. Some researchers have examined effects of habitat on abundance of black sea bass. Malek et al. (2014) stated that black sea bass are sensitive to physical changes in the seafloor, such as those caused by structural development.

Schweitzer and Stevens (2019) reported an increase in abundance of black sea bass in areas where colorful sea whips (*Leptogorgia virgulata*) were located on the seafloor. Although the intent of our study was not to determine whether environmental disturbances associated with the construction of offshore wind structures could affect black sea bass, offshore wind structures can serve as artificial reefs, and therefore habitats, for these fish given the physical characteristics of such structures. In this study, we investigated the effects of habitat type (natural versus artificial reef structures) on the demographics (size, age, and size at age) and feeding history (gut contents and stable isotope composition) of black sea bass to test the hypothesis that type of reef habitat does not affect these aspects of the biology and ecology of this species.

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Sampling occurred offshore of Maryland, near Ocean City, from coastal waters in spring and summer to near the continental shelf edge in the fall and winter (Moser and Shepherd, 2009; Fabrizio et al., 2014). During the period of this study, May–November, black sea bass associate with hard-bottom structures, henceforth referred to as reefs, with high rugosity at depths <28 m (Fabrizio et al., 2013). Black sea bass are protogynous hermaphrodites, reaching a maximum size of about 45 cm in standard length (2.3 kg) and longevity of >10 years (Lavenda, 1949; Wenner et al., 1986; Provost et al., 2017).

Black sea bass have a broad benthic diet, with some degree of dietary specialization on decapod crab species (particularly *Cancer* spp.), but the diet also includes a variety of other invertebrates and small fish species (Smith and Link, 2010). Byron and Link (2010) found ontogenetic diet shifts in black sea bass from mostly polychaetes and arthropods to fish, when the black sea bass are between 9 and 14 cm in total length (TL). However, considering that black sea bass prefer reef habitats, and even have exhibited fidelity to their selected habitats on an annual basis (Moser and Shepherd, 2009; Fabrizio et al., 2013; Secor et al., 2019), our focus was on the effect of reef habitat substrates, whether artificial or natural. In other studies, habitat of black sea bass has been related to feeding dynamics (Steimle and Ogren, 1982; Sedberry, 1988; Lindquist et al., 1994; Steimle and Figley, 1996), but no study has involved characterization of life history, feeding, and stable isotope signatures as they relate to habitat. For example, if habitat type affects prey type and growth, changes in available habitat could affect the productivity of a fishery that targets black sea bass, and effects on fishery productivity may be dynamic and widespread at the large scale of wind energy development planned in this region (Methratta et al., 2020).

Feeding history has been inferred from values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, both measures of stable isotope composition, in the tissues of fish (Araújo et al., 2007; Becker et al., 2007; Bauchinger and McWilliams, 2009), but in our study, we employed a tissue source that is relatively novel for black sea bass. For 2 commonly used sources, muscle and liver tissues, isotopic turnover occurs over months and weeks, respectively. In mucus, a tissue that can be collected in a way that is not lethal, turnover times for stable isotope signatures apparently are quite variable.

Church et al. (2009) noted the utility of using external mucus to determine short-term (30–36 d) turnover in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in juvenile steelhead trout (*Oncorhynchus mykiss*) but recommended sampling multiple tissues, and Maruyama et al. (2017) reported a much longer turnover (200 d) for these signatures in mucus samples of 5-year-old Japanese catfish (*Silurus asotus*). Results from a study by Ibarz et al. (2019) support the notion that food ingestion affects mucus exudation in gilthead bream (*Sparus aurata*) in short time intervals (≤ 24 h). Ibarz et al. (2019) found some enrichment at 12 h but did not find a significant difference at 24 h, suggesting that mucus sampling could be useful for examining “the effects of environmental challenges.” Winter et al. (2019) suggested that

epidermal mucus collected from live common carp (*Cyprinus carpio*) can replace the use of dorsal muscle for readings of stable isotope composition, on the basis of results from diet-switching lab experiments, but they cautioned that differences in tissue turnover rates are dependent on diet and need to be considered if used in field research. However, the reliability of mucus in tracking uptake of stable isotopes in wild fish has been quantified in few studies, and none, to our knowledge, have included testing mucus as a tissue fraction for readings of stable isotope signatures in black sea bass.

Sampling black sea bass at artificial and natural reefs offshore of Maryland over 2 years, we examined if habitat type was associated 1) with specific length, age, or size-at-age traits determined with otolith-based ages, 2) with snapshots of diets inferred from whole gut content analysis and compared to broad-scale diet data for the region, and 3) with feeding history inferred from analysis of stable isotope composition as sourced from fish liver, muscle, and mucus tissues and compared to isotopic values of common prey organisms in the region. We considered whether the 2 types of reef habitats were equivalent in terms of attracting and supporting black sea bass and what any differences might indicate about the biology and ecology of black sea bass on a continental shelf where wind energy systems may be developed.

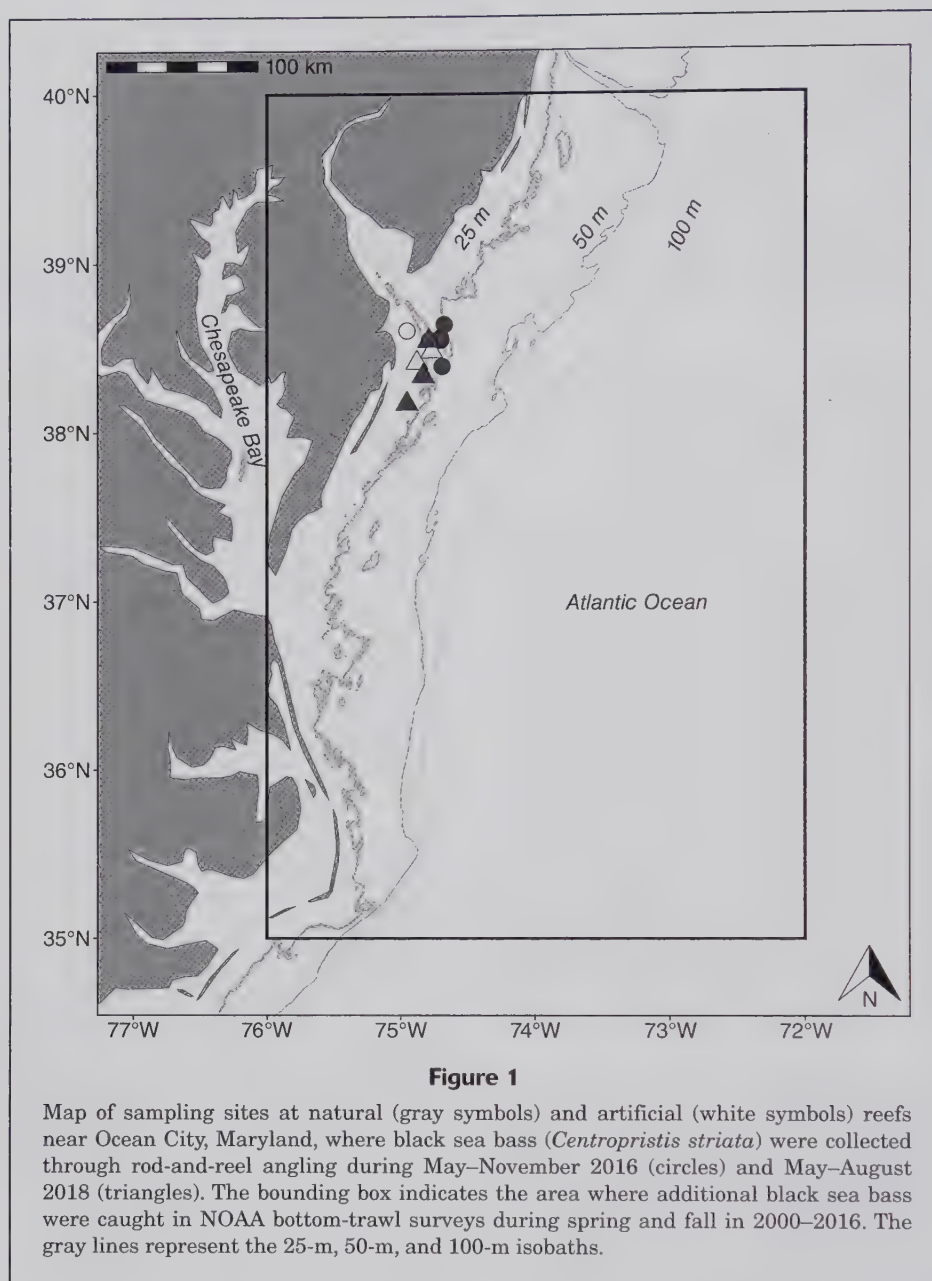
Methods and materials

Field sampling

Black sea bass were collected through rod-and-reel angling at natural and artificial reef habitats off the coast of Maryland, near Ocean City (Fig. 1), from May through November 2016 and from May through August 2018. Angling occurred during daylight hours, by using squid (*Loligo* spp.) as bait. All fish were immediately euthanized upon retrieval. Natural hard-bottom reef habitats consisted mostly of substrates of cobble and shell with a few soft corals, whereas artificial reef habitats were mostly shipwrecks and other metal structures. The locations and more detailed descriptions of the sampling sites appear in Supplementary Table 1 (online only).

Additional stomach content data were provided from black sea bass examined at sea, after capture from New Jersey to North Carolina during bottom-trawl surveys conducted by the NOAA Northeast Fisheries Science Center (Politis et al.¹). These comparative data were available for fish caught during spring (March–May) and fall (September–November) in 2000–2016 at latitudes between 35°N and 40°N (Fig. 1). Black sea bass were measured for size (in TL), and sex was determined macroscopically. Stomach

¹ Politis, P. J., J. K. Galbraith, P. Kostovick, and R. W. Brown. 2014. Northeast Fisheries Science Center bottom trawl survey protocols for the NOAA Ship *Henry B. Bigelow*. NOAA, Natl. Mar. Fish. Serv., Northeast Fish. Sci. Cent. Ref. Doc. 14-06, 138 p. [Available from website.]



contents were identified macroscopically and recorded to the lowest possible taxonomic level (Smith and Link, 2010). The methods of measurement and of stomach content and sex determination were consistent for fish sampled in bottom-trawl surveys and for the fish caught during the rod-and-reel angling of this study.

To compare stable isotope composition, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, for samples of potential prey items to those for fish tissues, whole organisms representative of potential prey species were fished in the fall of 2018 in both natural and artificial reefs, by using a small-mesh otter trawl to sample benthic organisms from flat-bottom habitats adjacent (<1 km) to the sites sampled in 2018. Specimens of the known species collected during tows of the trawl were retained.

Fish and tissue processing

Upon capture by angling, fish were immediately placed in individual plastic freezer bags to prevent mucus contamination and immersed in ice. Samples were subsequently frozen at -27°C (-80°F) as recommended for minimal effect on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in contrast to other methods (Bosley and Wainright, 1999; Kaehler and Pakhomov, 2001). In the laboratory, fish were measured for TL (to the nearest 1 cm) and weighed (to the nearest 1 g), and sex was determined by the examination of gonad tissues. Fish that were not clearly identifiable as male or female after dissection were categorized as either transitional or unknown.

Stomachs were initially placed in 10% formaldehyde for a minimum of 2 weeks and transferred to 70% ethanol for preservation until further sorting of prey items. Prey items were removed from stomachs of black sea bass, weighed to the nearest 0.01 g, and identified to the lowest possible taxonomic level.

Mucus, liver, and muscle samples were prepared for stable isotope analysis. Livers were removed, white muscle tissue was excised from an area directly above the pectoral fin, and both types of tissue samples were immediately frozen at -27°C . In 2018, a subsample of fish collected that year was selected to test mucus for stable isotope analysis with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Also in 2018, otoliths were collected from and used to age fish sampled in that year.

Age and growth

Otoliths (sagittae) were taken from fish, when possible, rinsed with fresh water to remove excess tissue, and stored dry in envelopes. Following techniques adapted from Penttilä and Dery (1998), whole otoliths were placed in a black dish containing a solution of 5% dish soap, and whole annuli were counted by using reflected light and a dissecting microscope. A subset of paired ages of fish from a second reader were used for quality control, by following the guidance of McBride (2015) and by using the package ALKr (vers. 0.5.3.1; Loff et al., 2014), with code developed by Ogle (2016), in statistical software R, vers. 3.4.0 (R Core Team, 2017). Ages assigned to fish by using otoliths were compared between readers by using percentage of agreement and Chang's coefficient of variation (Chang, 1982), and bias was assessed by using Bowker's test (Bowker, 1948). Between-reader precision was good (percentage of agreement=80%, Chang's coefficient of variation=4.98%), and there was no significant bias between readers (Bowker's test: $P=0.25$). For each otolith, final monthly age was assigned as follows (Wuenschel et al.²):

$$A + M - (4 / 12), \quad (1)$$

where A = the number of annuli; and
 M = the month of collection.

The fraction (4/12) is used to assume a spawning date in April.

Growth was examined by using a multinomial model of size at age in the R package FSA (vers. 0.818; Ogle et al., 2018) developed by Ogle (2016). Size-at-age relationships were not separated by sex because the black sea bass is a protogynous hermaphrodite (Hood et al., 1994). Instead,

the covariate used to test if size at age differed was *habitat type*. To determine whether fish sizes at habitat were significantly different, we used several tests, including a Student's t -test, which can be used to determine whether the mean sizes of the population are different. A Kolmogorov–Smirnov test, a nonparametric test to compare 2 sample distributions, was also used to help compare age at length based on our samples.

Stomach analysis

A prey-specific index of relative importance (PSIRI), expressed as a percentage, was calculated as follows (Brown et al., 2012; Varela et al., 2017):

$$PSIRI = (\%FO_i \times [\%PN_i + \%PW_i]) / 2, \quad (2)$$

where $\%FO_i$ = (number of stomachs with prey item i / total number of non-empty stomachs)100;

$\%PN_i$ = (number of prey item i / total number of prey items)100; and

$\%PW_i$ = (weight of prey item i / total weight of all prey items)100.

The PSIRI was chosen because the values are additive in respect to taxonomic level, and that additive approach facilitates the comparison of prey composition of fish stomach contents between the habitat types (Brown et al., 2012). Chi-square tests of independence were used to determine if significant differences existed between diets of fish at artificial and natural habitats, with response variables of *habitat type*, *size*, or *sex*. The PSIRI was used to compare prey composition in stomach contents between fish collected for this study and those collected during NOAA bottom-trawl surveys. Fish were categorized as small (0–25 cm TL), medium (25–50 cm TL), and large (>50 cm TL) on the basis of sizes used in Byron and Link (2010). No large fish were sampled by using rod-and-reel gear.

Stable isotope analysis

Muscle and liver samples were dried for 48 h in an oven at 65°C , homogenized with a mortar and pestle, and enclosed in tin capsules before being shipped for analysis. Samples not sent on the day that they were processed were stored in a desiccator to prevent hygroscopic weight gain. Muscle and liver samples were shipped to the Stable Isotope Facility of the University of California, Davis, for stable isotope analysis and processed with an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer. Samples were prepared in accordance to the University of California, Davis, protocol regarding sample preparation for analysis of ^{13}C and ^{15}N in solids by using an elemental analyzer and isotope ratio mass spectrometer (UC Davis³).

² Wuenschel, M. J., G. R. Shepherd, R. S. McBride, R. Jorgensen, K. Oliveira, E. Robillard, and J. Dayton. 2012. Sex and maturity of black sea bass collected in Massachusetts and Rhode Island waters; preliminary results based on macroscopic staging of gonads with a comparison to survey data. A working paper for SARC 53- [sic] black sea bass data meeting, September 2011. In 53rd Northeast regional stock assessment workshop (53rd SAW) assessment report. NOAA, Natl. Mar. Fish. Serv., Northeast Fish. Sci. Cent. Ref. Doc. 12-05, p. 529–546. [Available from website.]

³ UC Davis (Regents of the University of California, Davis). 2023. Carbon and nitrogen in solids: carbon (^{13}C) and nitrogen (^{15}N) sample preparation. UC Davis Stable Isot. Facil. Last updated 2 May 2023. [Web page, accessed June 2016.]

Samples of mucus were treated in accordance with methods adapted from Church et al. (2009). Fish were removed from the freezer, which was set to -27°C , and still in their respective bags, thawed for 5 min or until mucus was present on the epidermis of the fish. Mucus was gently scraped from the dorsal side of the fish and placed into a glass scintillation vial, and then the collected mucus was rinsed 3 consecutive times with 5 mL of reverse osmosis water. Samples were shaken between each addition of reverse osmosis water. The filtrate of mucus and water was then passed through a 5- μm polycarbonate filter. A final rinse of 5 mL of reverse osmosis water was passed through the filter before the filtrate was decanted into a 50-mL plastic test tube. Subsequently, the filtrate was frozen at 0°C for at least 24 h and then cryodesiccated for 48 h in a lyophilizer. A spatula was used to mix the sample, which was then weighed, enclosed in tin capsules, and shipped to the Stable Isotope Facility for stable isotope analysis as described in the previous paragraph.

Results from analysis of stable isotope composition at the University of California, Davis, were reported in δ notation, scaled in parts per thousand (‰), which can be expressed in the following equation (adapted from Hayes, 2004), originally introduced by McKinney et al. (1950):

$$\delta^AX_{\text{STD}} = ([^AR_{\text{Sample}} / ^AR_{\text{STD}}] - 1)1000, \quad (3)$$

where δ^AX_{STD} = “the abundance of isotope A of element X in a sample relative to the abundance of that same isotope in a [. . .] standard” (Hayes, 2004);

$^AR_{\text{Sample}}$ = the ratio of the abundance of isotope A in a sample; and

$^AR_{\text{STD}}$ = the ratio of the abundance of isotope A in an isotopic standard.

Stable isotope composition was compared by using 2-way analyses of variance to determine whether significant differences existed between the 2 habitat types or among fish sizes and whether an interaction of habitat type and fish size existed. The assumptions that the observations were independent, normally distributed, and had constant variance, as advocated by Ogle (2016), were met. The following linear model was the one tested by using analyses of variance:

$$D_T \sim (H_i \times S_j) + \varepsilon, \quad (4)$$

where D = either the $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ value;

T = tissue type;

H = habitat type;

S = size (TL in centimeters); and

ε = experimental error.

The analyses of variance were evaluated with the critical P -value set at 0.01, less than the standard threshold of 0.05, to account for some of the multiple comparisons inherent in the use of multiple tissue types. Scatterplots with ellipses were used to display overlap of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ data, with ellipses representing the 95% confidence intervals. Ellipses were created by using the function

stat_ellipse in the R package ggplot2, vers. 2.2.0 (Wickham, 2016).

To compare isotopic niches between fish sampled at artificial habitats and those sampled at natural habitats, the R package SIBER (vers. 2.1.0; Jackson et al., 2011) was used. In part of this package, Bayesian statistics are used to make models that help display isotopic niches among groups in a community. The SIBER package is used to compare isotopic niches between groups of samples, as advocated by Suca and Llopiz (2017).

Results

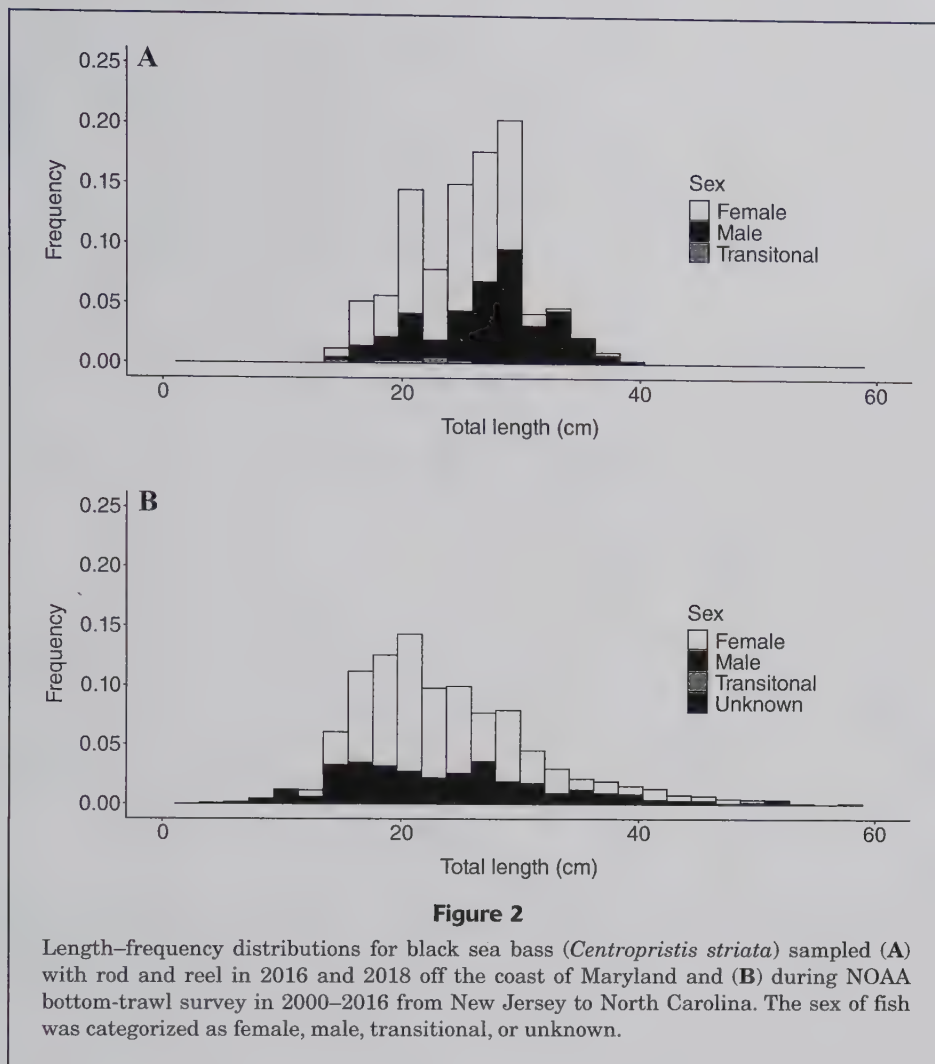
In this study, 407 black sea bass were caught: 107 fish in 2016 and 300 fish in 2018 (Suppl. Table 1) (online only). Fish were sampled from 2 types of reef habitats: 194 fish at artificial reefs and 213 fish at natural reefs. Of the 300 fish caught in 2018, 280 fish were used for age analyses. As expected for individuals of a protogynous hermaphroditic species, female black sea bass were caught more often than males. This pattern occurred for fish caught through rod-and-reel angling, with 235 females, 165 males, 6 transitional fish, and 1 fish of unknown sex, as well as for fish in the data from NOAA bottom-trawl surveys, with 874 females, 323 males, 2 transitional fish, and 105 fish of unknown sex (Fig. 2). As expected, males were larger than females. Among fish captured by using rod and reel, males had a mean size of 27.2 cm TL (standard error [SE] 0.41) and females had a mean size of 23.9 cm TL (SE 0.26) ($t=6.83$, $P<0.001$). In the data for fish captured during bottom-trawl surveys, the mean size of males and females was 26.7 cm TL (SE 0.46) and 23.7 cm TL (SE 0.24) ($t=5.89$, $P<0.001$). In terms of habitat, the size of fish sampled by using rod-and-reel gear did not differ between artificial and natural habitats (Kolmogorov–Smirnov test: $D=0.82$, $P=0.49$).

Age–length analysis

The estimated age of the fish caught for this study ranged from 2 to 6 years, with the age of the majority of the fish between 2 and 4 years (Fig. 3). Fish age did not differ between types of reef habitat, whether ages were estimated directly from otoliths (sample size [n]=280) (Student's t -test: $P=0.0015$) or, for a larger subsample of fish caught by using rod-and-reel gear, ages of some fish were estimated with an age–length key ($n=407$; Student's t -test: $P>0.11$).

Stomach content analysis

Arthropods dominated the diets of black sea bass at artificial and natural habitats by percent number, weight, and frequency of occurrence, with relatively higher PSIRI values for annelids and mollusks in natural habitats. A graphic display of PSIRI values for components of the diet of black sea bass is provided in Figure 4. Calculated PSIRI values for the prey categories of annelids, arthropods, fishes, and mollusks in stomach contents of fish caught at natural



habitats from 2016 through 2018 are 2.9%, 17.4%, 0.5%, and 11.1%, respectively, and the PSIRI values for fish caught at artificial habitats during that same period are 0.1%, 51.5%, 1.1%, and 2.3% (Fig. 4, Suppl. Table 2 [online only]). A full list of PSIRI values for prey items in the stomach contents of fish sampled is provided in Supplementary Table 2 (online only). Graphic representation of PSIRI values for prey items of the fish captured from artificial and natural habitats in 2018 can be found in Supplementary Figure 1 (online only).

The majority of arthropod taxa in the data from our stomach content analysis and in the data from NOAA bottom-trawl surveys were true (Brachyura) and hermit (Anomura) crabs (Suppl. Table 3) (online only). Annelids composed a higher percent weight (34%) but had a lower frequency of occurrence (0.07%) in the diets of black sea bass caught during bottom-trawl surveys than in those of black sea bass caught through rod-and-reel angling. For fish in the small and medium size classes, arthropods had the highest percent number, as shown in the graphic display of PSIRI values in Supplementary Figure 2 (online only). Approximately 40% ($n=83$) of the 205 fish caught at

natural habitats had empty stomachs, but only about 13% ($n=25$) of 193 fish from artificial habitats had stomachs that were empty. For fish with prey present in their stomachs, results of a chi-square test of independence indicate that there were no significant differences in prey consumption by black sea bass between artificial and natural habitats, when examining the relationships of prey number and fish size ($P=0.29$, $\chi^2=2.43$) or sex ($P=0.18$, $\chi^2=3.41$). A full list of prey items found in stomachs of fish based on data from rod-and-reel angling and from bottom-trawl sampling can be found in Supplementary Table 3 (online only).

Stable isotope analysis

For all tissue types (liver, muscle, and mucus), $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values overlapped between habitat types (artificial versus natural) and available fish size classes (medium versus small) (Fig. 5). Isotopic values differed between habitats for both stable isotope signatures and for all 3 tissue types except for $\delta^{15}\text{N}$ in muscle samples (Table 1). In addition, isotopic values differed by fish size only for $\delta^{13}\text{C}$

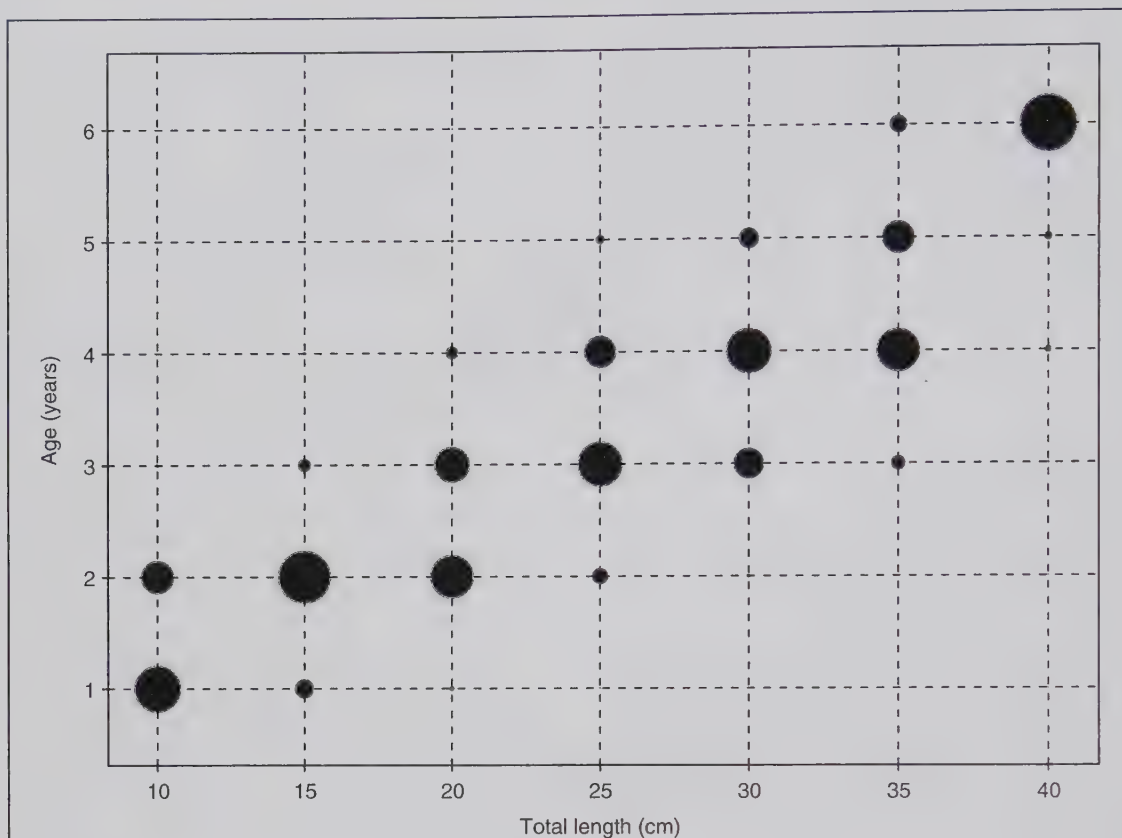


Figure 3

Length at age for 280 black sea bass (*Centropristis striata*) caught with rod and reel in 2018 off the coast of Maryland. Age and length did not differ between habitat types; therefore, samples caught at natural and artificial reefs were pooled. Bubble size is proportional to the relative number of fish per age and length interval.

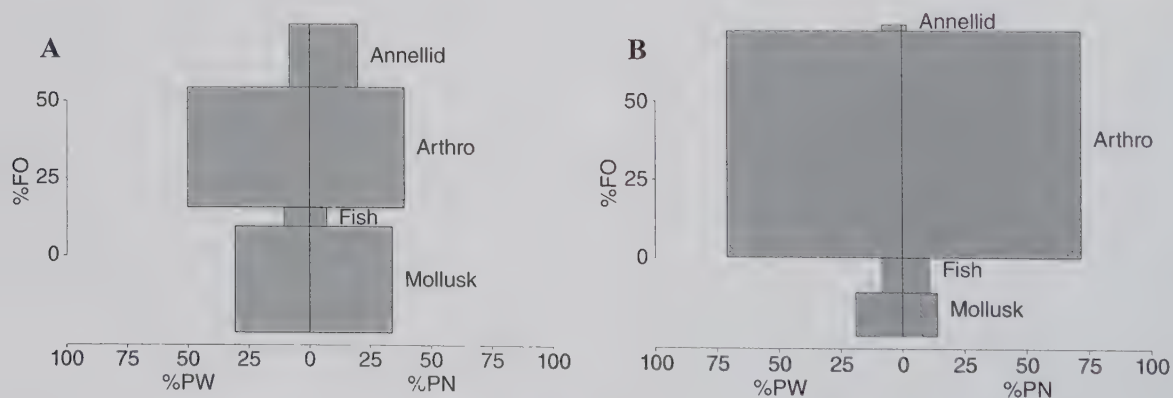


Figure 4

Values for components of the prey-specific index of relative importance (PSIRI) used to examine the diets of black sea bass (*Centropristis striata*) caught at (A) natural and (B) artificial reef habitats through rod-and-reel angling off the coast of Maryland in 2016 and 2018. Prey items include annelid, arthropod, fish, and mollusk taxa. The PSIRI is based on the following components: percent number (%PN), weight (%PW), and frequency of occurrence (%FO).

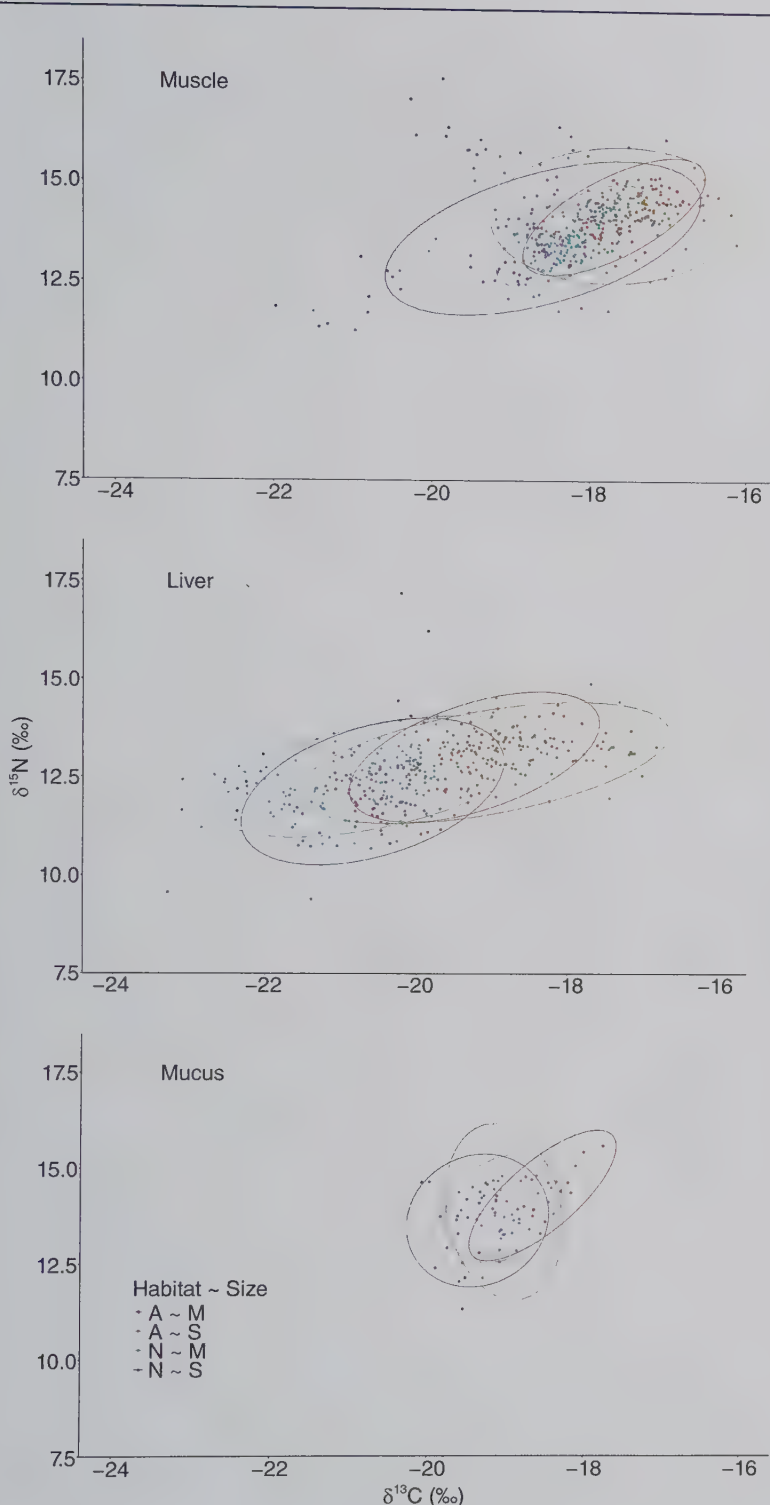


Figure 5

Stable isotope signatures, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, in muscle, liver, and mucus tissues of medium (M) and small (S) black sea bass (*Centropristis striata*) caught in natural (N) and artificial (A) reef habitats off the coast of Maryland in 2018. Fish were categorized as small if they measured 0–25 cm in total length (TL) and medium if they measured 25–50 cm TL. Ellipses represent 95% confidence intervals.

in mucus tissues, and they differed as an interaction of habitat and size only for $\delta^{13}\text{C}$ in muscle and mucus tissues.

Results from visual analysis for potential prey items sampled in 2018 indicate a lack of direct overlap in stable isotope signatures with the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for sand dollars (*Echinarachnius* spp.), mussels (*Mytilus* spp.), clams (*Merccenaria* spp.), and scallops (*Pecten* spp.), indicating that the black sea bass is positioned at a higher trophic level.

For the SIBER analysis in which isotopic niches were compared between habitat types, the standard ellipse area is a proxy for isotopic niche and can be interpreted as the area that an ellipse would occupy on a Cartesian coordinate plane. The vertical length, representing the area, of each icon conveys the variability of the predator's prey consumption or how diverse a predator's diet may be in a food chain, with the assumption that this diet is reflected in the tissues sampled. The location of the plot symbol, as it relates to the y-axis, provides a general indication of where the animal feeds on a basic trophic scale, where an increase of one trophic level would be represented as an increase of 3–4‰² in the stable isotope signatures shown. The SIBER analysis results indicate small differences (<5‰² total) in the standard ellipse area, signifying isotopic niches in small and medium fish; these results also indicate inconsistency among tissue types (Fig. 6). Standard ellipse areas for liver and muscle samples were larger than those for mucus samples in a comparison of values between small and medium fish, regardless of habitat type. Isotopic niches between fish sampled at artificial and natural habitats did not differ greatly (<4‰²), but for both groups, values for liver samples were consistently the highest, followed by those for muscle and mucus samples.

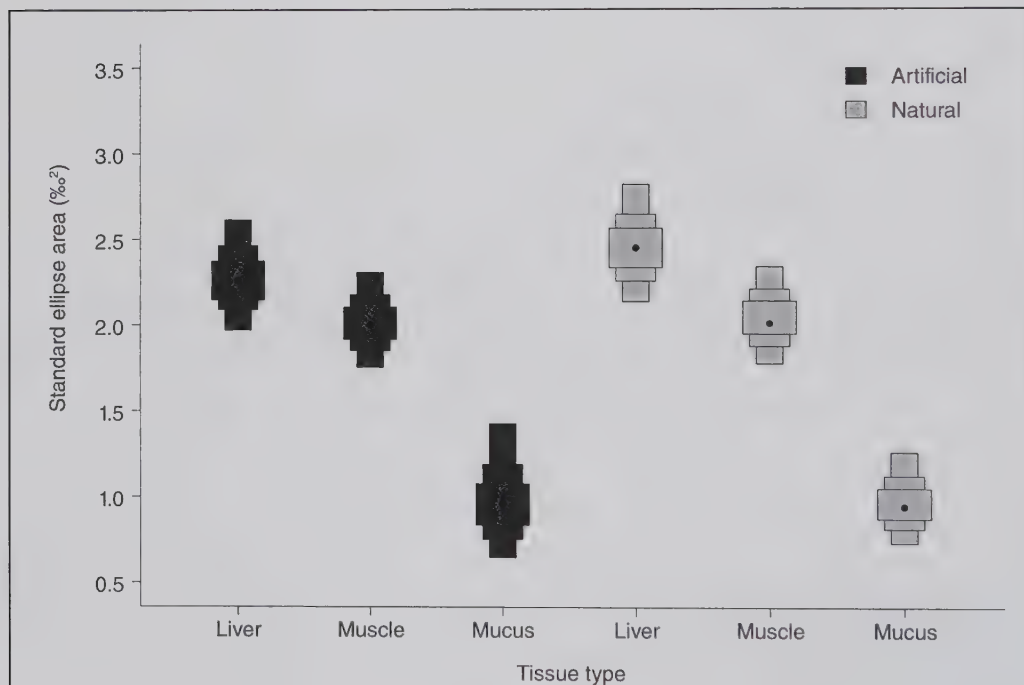
Discussion

Artificial reefs function similarly to natural reef habitats for black sea bass in marine waters off Maryland and New England, given that individuals sampled at sites of both habitat types were similar in several traits that we examined: age, length, length at age, and gut contents. The initial expectation of this

Table 1

Comparison of stable isotope signatures, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, in liver, muscle, and mucus tissues of black sea bass (*Centropomus striata*) caught in 2018 near Ocean City, Maryland, based on analysis of variance for habitat type (natural versus artificial) and size class (small [0–25 cm in total length] versus medium [25–50 cm in total length]) and for the interaction of those 2 variables and the interaction between habitat type and consumer level (predator versus prey). *P*-values from analysis of variance were assessed at 2 levels of significance: $P < 0.001$ and $P < 0.01$.

Variable	<i>P</i> -value					
	$\delta^{15}\text{N}$			$\delta^{13}\text{C}$		
	Liver	Muscle	Mucus	Liver	Muscle	Mucus
Habitat type	<0.001	<0.001	0.07	<0.001	<0.001	<0.001
Fish size class	0.01	0.99	0.03	0.74	<0.001	<0.001
Habitat $\times$ size	0.88	0.79	0.81	0.92	<0.001	0.54
Habitat type $\times$ consumer level	<0.001	<0.001	<0.001	<0.01	0.28	<0.01

**Figure 6**

Standard ellipse areas, by tissue type and corrected for small sample size, for small (0–25 cm in total length [TL]) and medium (25–50 cm TL) black sea bass (*Centropomus striata*) sampled at natural and artificial reef habitats off the coast of Maryland in 2018. The lines on each box represent the 95%, 75%, and 50% credible intervals. The standard ellipse area is a proxy for isotopic niche, the location of the plot symbol in relation to the y-axis indicates the general trophic level of black sea bass based on stable isotope analysis of tissues, and the vertical length of each box indicates variability in prey consumption.

study was that habitat differences might drive variance in diet and subsequent differences in size or age, as suggested by Lawson et al. (2018). If black sea bass had a more calorically rich diet (e.g., one composed primarily of fish) at one habitat—but a calorically poorer diet (e.g., one

composed of primarily crabs) at another habitat—this difference in diet could have affected fish size, particularly size at age, between habitats. The interactions between feeding and growth, as well as survival, are in fact complex (Goodrich and Clark, 2023); therefore, it was not

surprising that we did not find evidence of such a simple postulation.

Nonetheless, values of stable isotope composition differed between fish of both size classes captured at natural and artificial habitats. There were notable differences in habitat traits and prey composition based on data from otter trawl collections near sampling habitats: natural habitats consisted mostly of large shell and cobble, with sea robins (*Prionotus* spp.) and squid egg mops, and otter trawl hauls at natural habitats mostly consisted of shrimp (Crangonidae) and small shells.

As expected, arthropods, a common prey item for black sea bass, dominated gut contents of fish from focused rod-and-reel sampling and broad-scale bottom trawling. We did not observe an ontogenetic diet shift from invertebrates to finfish, as reported by Byron and Link (2010); however, black sea bass in the large size class (>50 cm TL) were not present in the habitats that we sampled. We did notice a higher proportion of empty stomachs at natural habitats, but this trend was not associated with slower growth of black sea bass.

Analysis of stable isotope composition was more sensitive than analysis of gut contents alone, indicating trophic differences between natural and artificial reef habitats for the fish size classes evaluated. Significant differences in values of $\delta^{13}\text{C}$ in liver samples between habitat types may indicate that fish at different reef habitats frequently prey on items with distinctive $\delta^{13}\text{C}$ values. Specifically, fish caught at artificial reefs had higher $\delta^{13}\text{C}$ values, indicating that they feed on prey from more littoral or benthic sources, such as sand dollars or snails, instead of pelagic prey, such as smaller finfish. Yet this distinction was not reflected in our diet analyses, or in data on prey types from NOAA bottom-trawl surveys. The cause of this apparent disconnect in results between analysis of stable isotope signatures and analysis of stomach contents at the 2 habitat types is challenging to assign. Isotopic turnover times in wild fish would likely vary, considering that wild fish may have a wide breadth of prey options, a need to forage, and arguably slightly higher metabolic demands, which can alter turnover time.

In mucus samples, $\delta^{13}\text{C}$ values were significantly different between habitat types, but $\delta^{15}\text{N}$ values were not. The former result was consistent with findings for the other tissues tested, indicating that it is possible to use mucus, which can be sampled in a nonlethal way, for black sea bass in future isotopic studies. The use of fish mucus for stable isotope analysis has great potential, but this approach would benefit from experimental validation. Altering the stable isotope signatures of black sea bass in a controlled diet-switching experiment, in which food amounts (offered and consumed) and growth would be tracked from a known fish age until the end of the experiment, could help verify how long it takes for isotopic signatures to change in certain tissues, which food items have the greatest effect on signatures, and how certain foods affect growth by isolating the variable of diet. In fact, the black sea bass is a suitable model species for this research because of its history of being reared in

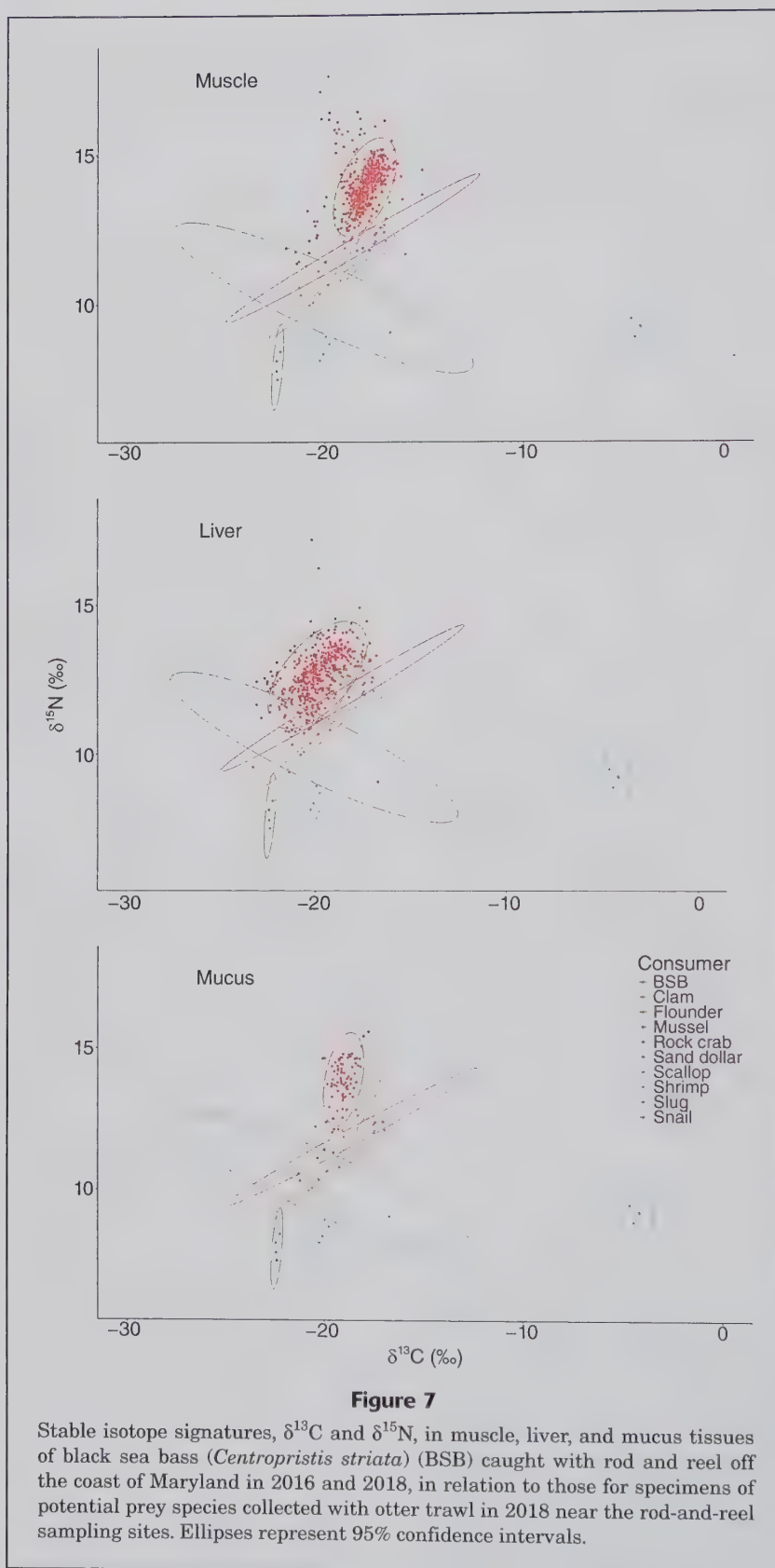
aquaculture facilities (Perry et al., 2007). The results presented herein (Fig. 7) can be used to inform selection of prey items for a prey-switching experiment over a season of growth.

Conclusions

In this study, black sea bass were found to feed and grow at identical rates at natural and artificial habitats, indicating that, from an ecological perspective, artificial reefs function in a similar manner as natural reefs as habitat for this species. Therefore, we conclude that risks of habitat alteration, or the addition of artificial habitat structures, associated with construction of wind energy systems should be relatively low for this common species on the continental shelf in this region. Nonetheless, the differences between habitat types in the stable isotope composition of tissue samples indicate functional differences in system energy flow that should be investigated further, not only for black sea bass but also for marine species that may also be affected by climate change or offshore wind energy development (Kleisner et al., 2017)—if we are to explain and predict the response of the many other such species in this region, we need to include them as well.

Resumen

El serrano estriado (*Centropristis striata*) se alimenta de presas epibentónicas, sobre todo decápodos, y se vuelve más piscívora a medida que crece, una tendencia que tiene consecuencias energéticas para su crecimiento. En las próximas décadas, la construcción de sistemas de energía eólica expone al serrano estriado a una manipulación a gran escala de los hábitats de la plataforma continental en el noreste de Estados Unidos. En este estudio, probamos los efectos del tipo de hábitat (hábitats de arrecifes naturales versus artificiales) en el tamaño, la edad, el crecimiento, la dieta y la dinámica trófica del serrano estriado, utilizando 407 especímenes recolectados con caña y carrete cerca de Ocean City, Maryland, en 2016 y 2018. No hay diferencias significativas en la longitud, edad, talla a la edad o dieta de los peces atribuibles al hábitat. La composición de isótopos estables ($\delta^{13}\text{C}$ y $\delta^{15}\text{N}$) en muestras de 3 tipos de tejidos (hígado, músculo y mucus) varía significativamente según el hábitat, excepto los valores de $\delta^{15}\text{N}$ en las muestras de mucus. En todos los tipos de tejidos, los valores de $\delta^{13}\text{C}$ son más elevados en los peces de hábitats artificiales, lo que indica una alimentación más litoral que pelágica. Concluimos que, aunque ambos tipos de hábitats arrecifales son ecológicamente equivalentes como fuente de crecimiento y dieta para el serrano estriado, las diferencias en las señales de isótopos estables indican diferencias funcionales en el flujo de energía del sistema que deberían investigarse más a fondo en esta y otras especies marinas que se verán afectadas por el desarrollo de la energía eólica marina.



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Dates should be written as follows: 11 January 2025. Measurements should be expressed in metric units, for example, "58 metric tons (t);" if other units of measurement are used, please make this fact explicit to the reader. Use numerals, not words, to express whole and decimal numbers in the general text, tables, and figure captions (except at the beginning of a sentence). For example, "We considered 3 hypotheses. We collected 7 samples in this location." Use American spelling. Refrain from using the shorthand slash (/), an ambiguous symbol, in the general text.

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For our journal, the word *aging* is used to mean both age determination and the aging process (senescence). Authors should make clear which meaning is intended where ambiguity may arise.

- *Fish and fishes*

The plural of the word *fish* (a collective noun that implies individuals without regard to species) is *fish*.

Example: The *fish* were collected by trawl net.

Example: The numbers of *fish* collected that season were less than the numbers from previous years.

The plural for *fish species* is *fishes* (a contrived plural used by taxonomists to mean several or more *fish species*) or one can use *fish species* (which is preferred in this journal for clarity across disciplines).

Example: The *fishes* of Puget Sound [biodiversity is implied] or

Example: The *fish species* of Puget Sound [preferred plural for clarity across disciplines].

- *Crab and crabs, squid and squids, etc.*

The plural of the word *crab* (i.e., many individuals without regard to species) is *crab*.

Example: The *crab* were sorted by weight.

Example: Many red king *crab* were dying [Many individuals of one species of crab.]

The plural of *crab species* is *crabs* (a word used by taxonomists) or *crab species* (the latter is preferred in this journal for clarity).

Example: These *crabs* were selected for treatment. [Different crab species are implied.]

Example: These *crab species* were selected for treatment. [Preferred word choice for clarity.]

Example: Snow *crabs* are found throughout the North Pacific Ocean and Bering Sea. [There are 2 species of snow crab; therefore the word *crabs* can be used here.]

Example: Two species of snow *crab* are found throughout the North Pacific Ocean and Bering Sea. [Preferred usage for clarity.]

Example: Three crabs were selected for treatment. [3 species of crab are implied.]

Example: Three crab species were selected for treatment. [Preferred word choice for clarity.]

- We use *fisherman* and *fishermen*, not *fisher* and *fishers*, in this journal. One can always use crew member, vessel operator, and angler (the latter for recreational fishing).

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When the singular common name of a species represents the entire class or group to which it belongs, use the definite article.

Example: Only one species of the genus *Salmo* is found in the Atlantic Ocean—the Atlantic salmon (*Salmo salar*).

Example: The sonic emissions of the bottlenose dolphin are complex.

For plural common names, this rule does not apply.

Example: Chinook salmon are found throughout the Pacific Ocean.

Example: Bottlenose dolphins are found in temperate and tropical waters.

- **Sex**

For the meaning of male and female, use the word *sex*, not *gender*. Do not write, “fish were sexed.” Write, “sex was determined.”

- **Participles**

As adjectives, participles must modify a specific noun or pronoun.

Example: Using mark-recapture methods, these scientists determined the size of the population. [Correct. The participle *using* modifies the word *scientists*.]

Example: These scientists, based on the collected data, concluded that the mortality rate of these fish had increased. [Incorrect. The scientists were not based on the collected data.]

Example: These scientists concluded, on the basis of collected data, that the mortality rate of these fish had increased. [Correct. The offending participle has been eliminated and an adverbial phrase modifies the verb *concluded*.]

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